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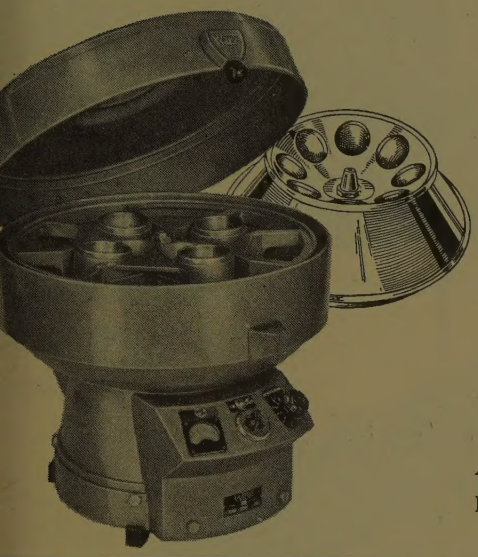

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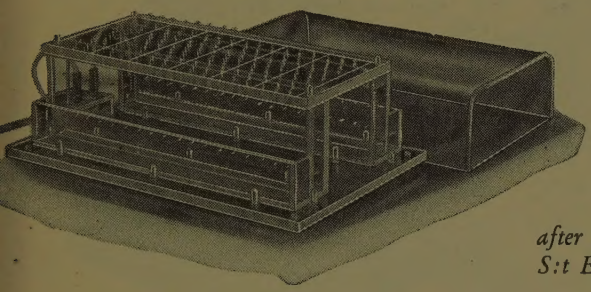

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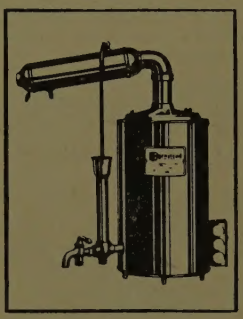

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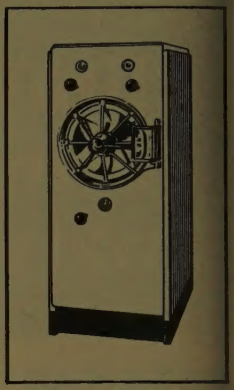

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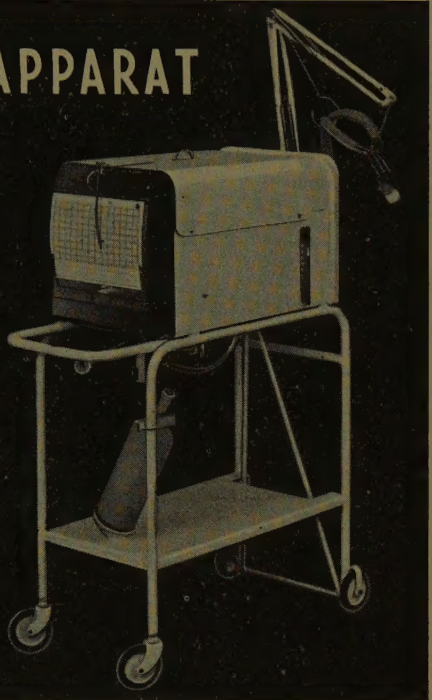

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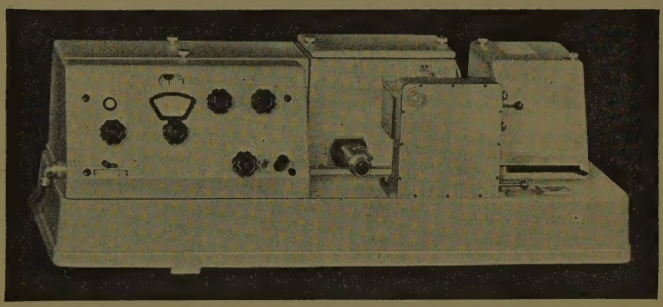

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NORMAL VALUES FOR PLASMA FIBRIN IN THE AGE GROUP 20—50 YEARS

By I. PERSSON

From the Nørre Hospital and the Danish Army Medical Service, København, Denmark

(Received for publication May 25, 1955)

INTRODUCTION

The purpose of this work is to endeavour throw light on the question of normal sma fibrin values in persons between 20 1 50 years of age.

Earlier statements (Cohn, 1947; Cullen van Slyke, 1920; Foster, 1924; Gram, 21; Halse, 1948; Ham & Curtis, 1938; gram, 1952; Jürgens, 1938; Klimesch & eltmann, 1927; Lasch, 1932; McLester, 22; Persson, 1955; Pfeiffer, 1897; Schultz, 53; Starlinger & Winands, 1928; Wester- n, Theorell & Widstrøm, 1931; Wootton King, 1953) concerning normal fibrin ues in adults differ rather widely. This y in part be due to the fact that the age l sex distribution in the persons examined been different. Gram (1921) states that erly persons somewhat more frequently, nough far from constantly, show the hest fibrin values. Lasch (1932) found t the fibrin values in elderly persons rose h the increase in years. Later Crane & Heyworth (1936) have carried out investigations concerning the fibrin in new born babies, and Starlinger & Winands (1928) in 20—30 year old persons. In his investigations Schultz (1953) found that the fibrin values rose with the increase in years in healthy persons. He chose, however, to base his investigations on a Kjeldahl analysis, and for this reason his results cannot be compared directly with the values obtained by the method most frequently used in this country, *i. e.* the weighing method.

EXPERIMENTAL MATERIAL

The investigations concerned 200 normal persons from three main groups. The first group comprised hospital personnel, the second personnel of the coastal defence, and the third blood donors. The groups appeared to be relatively adequate, as the two first mentioned groups were subject to fairly regular medical examination, with special reference to lung diseases, which are often the cause of the greater rises of the fibrin values (Schulz, 1953). Admittedly the third group was not subjected to regular medical examination, but as they were

regularly used as donors there was, however, a certain control on their state of health. Moreover, only persons who had presented no symptoms of any disease, and who had felt in good health, were included.

It was not possible in practice to take the blood samples while the patients were fasting; but instead it was decided to take them approximately. 1 hour after breakfast. However, as seen from Gram's investigations (1921) the 24 hour variation for normal fibrin values is not larger than that which may be due to experimental error. Likewise it has been shown by Ham & Curtis (1938) that high protein consumption over several days only causes a rise in the fibrin content within normal limits. Jürgens (1938) states, however, that the fibrin content is reduced during fasting, but further details are lacking.

In order to avoid influence of possible seasonal variations on the fibrin values the blood tests were evenly distributed over two years.

METHOD OF INVESTIGATION

It was decided to use the method of Gram (1921). He found in his investigations that the mean error for the fibrin values found, recorded as a percentage in plasma, was ± 0.011. In order to assess the precision with which it would be possible to carry out the investigations described below, repeated determinations have been made in 20 cases. The results are illustrated in Table I.

On the basis of the 20 determinations once repeated the standard error of the individual measurement is found to be 0.007, which is only $1/7$ of the dispersion of fibrin values from different persons presented below.

RESULTS

The distributions for the observed plasma fibrin values in point diagrams and on probit paper show that these may be considered approximately normal (Gaddum, 1945). It appears, though, as if they are not quite uniform, as the variation seems to rise with at the same time as there is a rise in mean value.

Table I. *Repeated determinations of plasma fibrin values.*

No. of patient	Plasma fibrin % I	Plasma fibrin % II	Difference fibrin %
1	0.34	0.34	0
2	0.28	0.29	0.01
3	0.44	0.44	0
4	0.28	0.28	0
5	0.35	0.33	0.02
6	0.32	0.31	0.01
7	0.28	0.28	0
8	0.30	0.31	0.01
9	0.31	0.31	0
10	0.34	0.36	0.02
11	0.28	0.28	0
12	0.32	0.32	0
13	0.45	0.44	0.01
14	0.31	0.30	0.01
15	0.28	0.30	0.02
16	0.28	0.27	0.01
17	0.38	0.38	0
18	0.42	0.40	0.02
19	0.29	0.29	0
20	0.28	0.28	0

Table II shows for the different age gr and for both sexes separately the follow n = number of observations, m = m value, s = standard deviation of the i vidual observation, d = difference betv the mean values in the different age gr and the difference between the mean va for both sexes in each of the three groups. The t values indicate the exter which the difference in question are s ficant. 1, 2, and 3 stars (*) respecti show that the values lie beyond the 5 cent—1 per cent, and 0.1 per cent — 1 It appears that the rise with age is disti significant. The mean value for the wo lies sligthly higher than that for the I However, the differences for each indiv age group are not significant, but by ad

Table II. *Plasma fibrin values in per cent for normal persons.*

	Women					Men					Women - Men	
	n	m	s	diff.	t	n	m	s	diff.	t	diff.	t
30	40	0.305	0.043			40	0.289	0.040			0.016	1.7
				0.035	2.7**				0.025	2.1*		
40	30	0.340	0.060			30	0.314	0.055			0.026	1.7
				0.082	5.4***				0.106	6.6***		
50	30	0.422	0.055			30	0.420	0.068			0.002	0.1
												3.5
											$\bar{t}$ =	2.06*

Table III. *Plasma fibrin values in fractiles.*

	Women				Men			
ge	Lower 1%	Lower 10%	Upper 1%	Upper 10%	Lower 1%	Lower 10%	Upper 1%	Upper 10%
—30	0.21	0.25	0.36	0.41	0.20	0.24	0.34	0.38
—40	0.20	0.26	0.42	0.48	0.19	0.24	0.39	0.44
—50	0.29	0.35	0.49	0.55	0.26	0.33	0.51	0.58

he t values a significant average t value btained. On the vhole, therefore, the rence between the mean values for ien and men is significant.

able III shows the estimated lower and r 1 per cent and 10 per cent fractiles e plasma fibrin values. Normally 98 per should be found between the lower and r 1 per cent fractile, and 80 per cent een the lower and upper 10 per cent ile.

DISCUSSION

ne fact that the plasma fibrin rises with and that on the whole it is highest in en, agrees with previous investigations ne *et al.*, 1936; Foster, 1924; Gram, ; Lasch, 1932; Persson, 1955; Schulz, ; Starlinger *et al.*, 1928). However, as ioned in the introduction, previous stigations have often shown mean values h differ rather considerably from each , even if the outer limits are almost the same, *i. e.* between 200 and 500 mg per cent in most cases.

The difference in the results may perhaps partly be ascribed to different methods of investigation, *e. g.* solubility (Wuhrmann & Leuthardt, 1938), ultracentrifugation (Svedberg & Pedersen, 1940), electrophoresis (Wuhrmann & Wunderly, 1947), Harvard fractionation (Cohn, 1947), refractometry (Starlinger, 1923), weighing method (Crane & Heyworth, 1936; Foster, 1924; Foster & Whipple, 1922; Gram, 1921; Ingram, 1952; Klimesch & Weltman, 1927; Lasch, 1932; Mc Lester, 1922; Persson, 1955; Starlinger & Winands, 1928, or Kjeldahl analysis (Crane & Heyworth, 1936; Cullen & van Slyke, 1920; Halse, 1948; Ham & Curtis, 1938; Pfeiffer, 1897; Schulz, 1953; Westergren *et al.*, 1931). Moreover, some of the previous investigations (Klimesch & Weltman, 1927; Pfeffer, 1897; Westergren *et al.*, 1931)) concerned a few patients only.

Sometimes the choice of normal material has been uncritical (Pfeiffer, 1897), as patients suffering from organic diseases were included. Experiments have moreover shown that identical methods of investigation of the same sample give different results when carried out in different laboratories (Wootton, King & Smith, 1951).

Apart from stating the mean values and standard deviations four levels for each age group have been included in the investigations here described, as illustrated by the tables, viz. the lower and upper 1 per cent fractiles. One reservation must be made with reference to the limits for the fibrin values thus calculated; they are associated with relatively great inaccuracy, as they depend upon both the mean values and the standard deviations, and the distributions seem to indicate that other relevant factors than age and sex ought also to be considered. In fact, certain investigations seem to indicate that other physiological conditions as well affect the plasma fibrin values. Menstruation (Schulz, 1953), pregnancy (Foster & Wipple, 1922; Jürgens, Bennhold, Kylin & Ruszńyak, 1938; Schulz, 1953), and hard physical labor (Jürgens *et al.*, 1938; Schulz, 1953) are thus said to influence the content of plasma fibrin. None of the persons examined were, however, pregnant, nor had they performed hard physical labour before the blood samples were taken. In the present investigations no consideration has been paid to the stage in the menstrual cycle at examination, as the case material was considered too small for such further grouping.

Several facts may be considered of importance in causing the plasma content of fibrin to change with age. For one thing the older age groups represent selected material cause they are composed of surviving viduals. Previous diseases, dietetic ha and other habits of living, apart from normal process of aging may probably a part. As there is strong individual v tion is does not seem unreasonable to sume that the variability of the valu larger in older than in younger person illustrated by a tendency in the resul the present investigations.

SUMMARY

Investigations on 200 normal per whose ages range between 20—50 years shown that the plasma fibrin rises with and is generally higher in women. W view to furthering the evaluation o experimental results the lower and u 1 per cent and 10 per cent fractiles been calculated in addition to mean v and standard deviations. The differenc the districtions for the fibrin values to indicate that in normal persons relvant factors than sex and age must be considered. Possible reasons why content of plasma fibrin varies with ag sex are discussed.

ACKNOWLEDGMENT

The calculations were performed by M Nyholm, actuary.

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A TITRIMETRIC METHOD FOR THE DETERMINATION O PLASMA AND RED BLOOD CELL CHOLINESTERASE ACTIV USING THIOCHOLINE ESTERS AS SUBSTRATES

By K.-B. AUGUSTINSSON

From the Institute of Organic Chemistry and Biochemistry, University of Stockholm, Stockholm, Sweden

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A great many methods have been described for the estimation of cholinesterase activity (Augustinsson, review in preparation). In almost all of these methods esters of choline (acetyl-, butyryl-, benzoyl-, or acetyl-β-methyl-choline) are employed as substrates. In most cases the techniques are based upon the determination of acid produced during the reaction. The idea of using esters of thiocholine as substrates for blood chlinesterases was first proposed recently by Meyer & Wilbrandt (1954). Such compounds, however, have been tested frequently during recent years in the histochemical detection of cholinesterases (Koelle, 1950). The use of thiocholine esters as substrates for blood cholinesterases in routine analysis has been anticipated in this laboratory for some time. The method worked out is, in principle, similar to that described by M & Wilbrandt. The advantages of the pr method are the use of 0.05 ml of whole b applied and dried on filter paper (Augus son & Heimbürger, 1953; Augustin 1955), a more suitable buffer solution, a more convenient titration technique.

MATERIAL AND METHODS

Principle

The cholinesterase activity of blood, 0.05 whole blood dried on filter paper, is estimat a titrimetric method using butyrylthiocholi substrate for the plasma butyrylcholines activity, and acetylthiocholine, in the precenc specific plasma cholinesterase activity. Durir reaction free SH groups are produced and are estimated quantitatively by iodometric titr The various steps during the complete proc are as follows:

1. Enzymic reaction:

$$(CH_3)_3\overset{+}{N}—CH_2—CH_2—S—COCH_3 \xrightarrow{H_2O} (CH_3)_3\overset{+}{N}—CH_2—CH_2—SH + HOCOC$$

2. Iodate and iodide added in excess:

$$IO_3^- + 5\,I^- + 6\,H^+ \longrightarrow 3\,I_2 + 3\,H_2O$$

3. Iodine oxidizes the free SH groups to SS groups:

$$2\,R—SH + I_2 \longrightarrow R—SS—R + 2\,I^- + 2\,H^+$$

4. Excess of iodine is titrated with thiosulphate.

Blood sampling

om the finger tip, exactly 0.05 ml of whole was taken up in a blood pipette (heparinized) hen applied on a filter paper and air-dried om temperature. Such blood samples can ored in the refrigerator for a month without f esterase activity (Augustinsson, 1955).

Buffer solution

rious buffer solutions have been tested. It ound that the barbital (veronal) buffer, simi- that introduced by Michel (1949) was pre- le especially when Mg^{2+} was added. The on recommended has the following composi- In 900 ml of distilled water the following compounds were dissolved: 4.1236 g sodium tal, 0.5446 g KH_2PO_4, 44.730 g KCl, and 5 g $MgCl_2 \cdot 6\,H_2O$; 15 ml of 0.1 N HCl was l and the volume made up to 1000 ml with ed water. The pH of this buffer should be (25° C).

Substrates

ck solutions of butyrylthiocholine iodide hCh) and acetylthiocholine iodide (AThCh) prepared by dissolving the pure compounds drochloric acid of pH 4.0, at which pH these ons are stable for months. The concentration e stock solutions was 0.1 *M*. Immediately be- use these solutions were diluted with four of the barbital buffer. The final concen- n of the substrates during activity measure- s was 5.0×10^{-2} *M* (total volume of the ion mixture, 2.00 ml).

elective inhibitor of plasma butyryl-cholinesterase

-diethylaminopropionyl)-phenothiazine hydro- ide, $C_{12}H_8SN{-}CO{-}CH(CH_3) \cdot N(C_2H_5)_2 \cdot$. This compound, here designated "Astra and synthezised by Dahlbom & Ekstrand), was found to be a selective inhibitor lasma butyrylcholinesterase when used in 10^{-3} *M* solutions; final concentration during ty determination, 1.65×10^{-4} *M*. At this con- ation there is no influence on the erythrocyte lcholinesterase activity (Table I).

Table I. *Effect of 10-(α-diethylamino-propionyl)-phenothiazine hydrochloride ("1397") on the human blood cholin-esterases. Acetylcholine iodide used as sub-strate. Activity expressed in μmoles of sub-strate hydrolysed in 30 minutes, corrections made for spontaneous hydrolysis of substrate. Gasometric method employed.*

Final molar concen-tration of «1397»	Plasma Control = 4.46 μmoles		Erythrocytes Control = 6.40 μmoles	
	μmoles hydro-lysed	Per cent inhi-bition	μmoles hydro-lysed	Per cent inhi-bition
5.0×10^{-4}	0	100	6.25	2
2.5×10^{-4}	0	100	6.25	2
1.65×10^{-4}	0	100	6.41	0
0.5×10^{-4}	0.13	97	6.40	0
0.1×10^{-4}	0.29	93	6.42	0

Other reagents

Trichloracetic acid: 10 per cent and 2 per cent aqueous solutions.

Potassium iodate, KIO_3; 0.01 N.

Potassium iodide, KI: 5 per cent aqueous solution.

Starch: 0.5 per cent solution.

Sodium thiosulphate, $Na_2S_2O_3 \cdot 5\,H_2O$: 0.005 N.

Procedure

The blood spot is cut out from the paper and placed in small pieces in a 10 ml Erlenmeyer flask. For the plasma butyrylcholinesterase assay 1.5 ml barbital buffer is added; for the erythrocyte acetylcholinesterase assay 1.4 ml barbital buffer and 0.1 ml of the solution of the phenothiazine derivative ("1397"). The mixtures were shaken in a water thermostat of 25° C for about 15 minutes before 0.5 ml of the substrate solution was added (at zero time). The reaction mixtures were shaken in the thermostat for 30 minutes after which period the reaction was stopped adding 4 ml of 10 per cent trichloracetic acid. The mixtures were filtered

and the precipitates washed with about 4 ml of 2 per cent trichloracetic acid. To the clear filtrates 1.00 ml of 0.01 N KIO_3, 2 drops of 5 per cent KI and 3 drops of 0.5 per cent starch solution were added. The mixtures were then titrated with sodium thiosulphate.

Dubblicate runs were made with each substrate. It was necessary to carry out a blank run without substrate. The spontaneous hydrolysis of the substrates, on the other hand, was negligible.

Calculation

If A = ml of thiosulphate required to reduce the excess iodine then μmoles of substrate hydrolysed = (meq KIO_3 — meq $Na_2S_2O_3$)1000 = (1.00 × 0.01 — A × 0.005)1000.

RESULTS

The titrimetric method in combination with the gasometric method. The enzymic hydrolysis of butyrylthiocholine and acetylthiocholine by 0.05 ml of whole blood (applied and dried on filter paper) was followed by the Warburg manometric technique

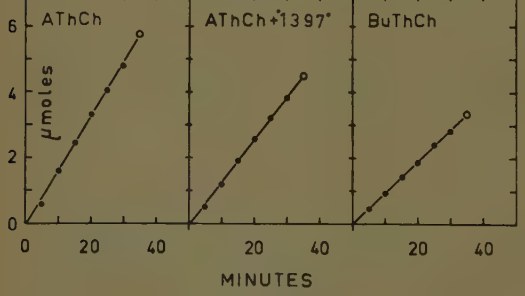


Fig. 1. Enzymic hydrolysis of acetylthiocholine and butyrylthiocholine by 0.05 ml of whole blood (applied and dried on filter paper). The reaction velocity (μmoles of substrate hydrolysed) was followed gasometrically (Warburg technique; values noted by "dots"); after 35 minutes the reaction was stopped by adding trichloracetic acid to the reaction mixture and the amount ("circles") of free SH groups titrated with iodate-thiosulphate.

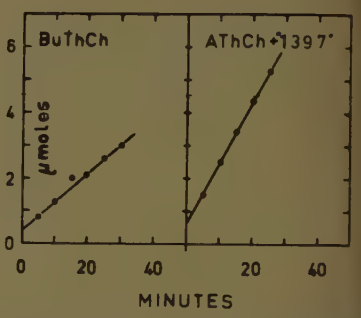
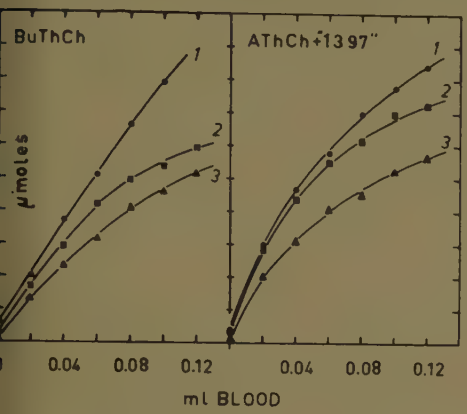


Fig. 2. Enzymic hydrolysis of thiocholine e by 0.05 ml of blood (dried on paper). Titrimetric method.

(in a bicarbonate buffer solution) (A stinsson & Heimbürger, 1953; Augustins 1955). The last readings of the manom were made after 30 minutes, the flasks disconnected from the manometers, and 35 minutes from zero time the reaction stopped by adding trichloracetic acid. the procedure described above, the rea mixtures were then titrated to determin amount of substrate hydrolysed. The re are recorded in Fig.1 which demonst the consistency of results obtained in paring titrimetric and gasometric met Further examples of this consistency, is especially valid in the case of pl cholinesterase, will be given below.

Reaction curve. When μmoles of subs were plotted against time a straight line obtained with the concentrations of en (blood) and substrates generally empl A typical example is shown in Fig. 2.

Enzyme activity as a function of blood centration. Various amounts of whole were applied and dried on paper an dried spots used as enzyme sources. It found (Fig. 3) that there was not alw strict proportionality between blood co



3. Cholinesterase activity as function of blood entration (whole blood dried on paper). Titri- ic method. 1: Barbital buffer; 2: Bicarbonate buffer; 3: Phosphate buffer.

ion and the reaction of the enzymic rolysis of the thiocholine esters used. ect proportionality was observed when ryrylthiocholine was used as substrate in arbital buffer solution. In other cases ct proportionality between reaction rate blood concentration was generally not d, especially when the selective butyryl- inesterase inhibitor ("1397") was pre- (Fig. 3). This makes the method less able for determining erythrocyte acetyl- inesterase.

arious buffer solutions. Instead of using barbital buffer solution, the composition hich was described above, a bicarbonate er (Augustinsson 1948) and a phosphate er (Meyer & Wilbrandt 1954) have tested in preliminary experiments. As in Fig. 3 the barbital buffer containing $^{+}$ gives the highest activity values. The lts obtained in a series with various buf- solutions with and without Mg^{2+} are rded in Table II.

Table II. *Comparison of the results obtained with various buffer solutions. Titrimetric method.*

Buffer	Mg^{2+}	µmoles of thicholine ester hydrolysed			
		BuThCh		AThCh+"1397"	
Bicarbonate	—	3.37	3.32	—	—
	+	3.57	3.63	6.53	6.65
Barbital	—	3.85	3.91	6.01	6.07
	+	4.07	4.07	6.60	6.65
Phosphate	—	2.81	2.86	4.64	4.62

Table III. *Accuracy of the titrimetric method.*

µmoles of substrate hydrolysed in 30 minutes	
BuThCh	AThCh+"1397"
3.43	6.12
3.28	6.14
3.47	6.09
3.41	6.12
3.34	6.07
3.41	6.03
Mean: 3.39	Mean: 6.10
S.D.: 0.07	S.D.: 0.04

Accuracy of the method. Table III lists the results of a series of determinations carried out with the same blood sample. The accuracy of the method determined in a larger series of experiments has been found to be ± 3—5 per cent.

Normal values obtained for human blood. There is a fairly satisfactory agreement between the plasma cholinesterase activity values obtained with the titrimetric and gasometric methods. The results obtained

Table IV. *Human (male) blood cholinesterase activity determined by the ti metric and manometric methods. Activityvalues expressed in moles of substr hydrolysed enzymically in 30 minutes by 0.05 ml of whole blood (on paper). BuC butyrylcholine iodide; MeCh: acetyl-β-methylcholine iodide.*

Blood sample No.	Titrimetric method		Gasometric method		BuThCh / BuCh	AThCh+' / MeCh
	BuThCh Plasma	AThCh+'1397' (Erythrocytes)	BuCh (Plasma)	MeCh (Erythrocytes)		
1	3.34	5.10	2.70	1.72	1.2	3.0
2	4.23	7.13	3.26	1.61	1.3	4.4
3	3.96	4.03	3.14	1.25	1.3	3.2
4	3.85	4.29	3.06	1.61	1.3	2.6
5	5.12	5.79	3.55	1.66	1.4	3.5
6	3.54	5.18	2.72	1.68	1.3	3.1
7	2.96	—	2.45	—	1.2	—
8	3.41	—	2.88	—	1.2	—
9	3.89	—	3.28	—	1.2	—
10	3.93	—	3.35	—	1.2	—
11*)	2.75	—	2.18	—	1.3	—
12**)	6.74	5.20	5.70	1.52	1.2	3.4

*) Ulcus ventriculi; **) Myasthenia gravis (female)

with a series of human blood samples are summarized in Table IV. This agreement is less satisfactory for the erythrocyte acetylcholinesterase activity.

COMMENTS

The most widely used methods for routine determinations of blood cholinesterase activity are the Warburg gasometric technique (Augustinsson, 1948, 1955), the Michel electrometric technique (Michel, 1949; Tammelin, 1953), and the continuous titration technique (Hall & Lucas, 1937, Morand & Laborit, 1948). The two first named techniques are probably those which give the best and most significant results, but they are rather laborious, need specially trained technicians, and the apparatus is expensive. The continuous titration technique has certain shortcomings; it is not as precise as the gasometric and electrometric techni and the reaction medium is altered du the determination procedure.

The method described in the present p should be of value as a routine metho blood cholinesterase determinations in cal laboratories. It uses a small amou whole blood and the technique is sir No special equipment and no special trai is necessary. The accuracy of the me however, is not as good as that of the metric technique but for clinical purpos is recommended primarily for rapid simple determination of the plasma ch esterase activity using whole blood. method is not as suitable in determinin erythrocyte acetylcholinesterase; its ap tion in this case is dependent on the d opment of a specific thiocholine ester substrate for this enzyme. The thioch

r that corresponds to acetyl-β-methyl-ine is not as specific for the erythrocyte ylcholinesterase as its oxygen analogue oelle, 1950) and in addition it is not lily available at the present time.

SUMMARY

A simple method of determining cholin-rase activity in whole blood is described. yrylthiocholine is used as a substrate for plasma esterase activity. Acetylthio-line, in the precense of a specific butyryl-linesterase inhibitor, is used when the erythrocyte esterase activity is determined. The SH groups produced during the sub-strate hydrolysis are titrated iodometrically.

ACKNOWLEDGMENT

I wish to acknowledge my indebtedness to Dr. Richard Dahlbom, Centrallaboratoriet, AB Astra, Södertälje, Sweden, for the gift of the phenothiazine derivative. Butyrylthiocholine iodide and acetylthiocholine iodide were kindly placed at my disposal by Messrs. Hoffmann-La Roche; for this generosity I want to express my sincere thanks.

I am greatly indebted to Miss Torhild Isachsen for skilful technical assistance.

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ADDENDUM

After the completion of the experiments pre-ed above a small amount of *acetyl-β-methyl-choline iodide* was made available through the l generosity of Messrs. Hoffmann-La Roche, le, the scientific department of which, on my uest, synthesised this compound. The only report known to the present author about this ester as a substrate for cholinesterases is that recently published by Koelle (1950). He showed in preliminary experiments that this ester is less specific for acetylcholinesterase (red blood cells) than is its oxygen analogue ("mecholyl", meta-

Table V. *Enzymic hydrolysis of acetyl-β-methylcholine (MeCh) and acetyl methylthiocholine (MeThCh) by cholinesterase (ChE) preparations. Gasome method, unless otherwise stated. Activity expressed in μmoles of substrate split in minutes (corrections made for non-enzymic hydrolysis).*

Substrate	Spontan. hydrolysis	Purfied human plasma ChE	Human erythrocyte ChE (hemolysate)	Whole blood on paper	Purified el tissue Ch
MeCh	0.15	0.41	1.92	1.36	2.37
MeThCh	0.20	2.14	4.40	4.02 4.05*)	12.95

*) Titrimetric method.

choline"); therefore, it was not satisfactory for use in histochemical differentiation of various cholinesterases (in contradistinction to butyrylthiocholine).

Preliminary experiments with acetyl-β-methylthiocholine iodide (MeThCh) confirmed the observation made by Koelle. The enzymic hydrolysis of this ester was compared with that of acetyl-β-methylcholine (MeCh) using the Warburg technique and various enzyme preparations. It was (Table V) that the thiocholine ester was hydr by the plasma butyrylcholinesterase at a signi rate and therefore is not suitable for assayin activity of erythrocyte acetylcholinesterase in blood. The thiocholine ester is split at a higher rate by acetylcholinesterases than oxygen analogue. The substrate concentration was 5.0×10^{-2} *M*.

IE DETERMINATION OF SERUM PROTEIN-BOUND IODINE BY ALKALI INCINERATION[1]

VALUES IN NORMAL SUBJECTS

By B. SKANSE and I. HEDENSKOG

rom the Department of Medicine and the Department of Clinical Chemistry, University of Lund, Malmö General Hospital, Malmö, Sweden

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n the past ten to fifteen years determina- of protein-bound iodine in the serum BI) has proved valuable in the investiga- of thyroid function. The methods avail- have, however, been to intricate for tine clinical use. Barker's (1948) distilla- method for the determination of serum tein-bound iodine aroused considerable erest. The later fairly simple alkaline ing method of Barker & Humphrey 50) and Barker, Humphrey & Soley 51) made the analysis of serum protein- nd iodine available as a routine labora- y procedure. For 68 euthyroid subjects ker *et al.* (1951) found a mean of 5.1 μg 100 ml with a standard deviation of μg per 100 ml. A clinical evaluation of ker & Humphrey's method was published Hallman, Bondy & Hagewood (1951). eir mean normal value, based on deter- ations in 37 normal subjects, was 5.4 μg 100 ml with a standard deviation of μg per 100 ml, a level in good agreement h that reported by Barker *et al.* and com- ible with those found by other investiga- s using different methods.

n 1952, when we substituted Riggs & n's more intricate permanganate acid ashing method for the alkaline incineration technique of Barker *et al.* (1950, 1951), we could not always obtain reproducible results. However, subsequent modification of certain steps resulted in a reliable and sensitive method. As this modification, which we have since used for more than 6000 analyses, appears to provide a useful routine laboratory test the method is described in detail below.

A modification of the final colorimetric determination of iodide in the method of Barker *et al.* was suggested by Brown, Reingold & Samson (1953). Grossmann & Grossmann (1955) proposed an alkali incineration method in which the colorimetric determination is made from a reaction that produces a stable color so that light transmission measurements are claimed to be made without color drift. As yet we have not succeeded in obtaining reproducible results by either of these methods.

METHOD

Principle

The method described below consists mainly of the following four steps: 1) precipitation and washing of the serum proteins, 2) dry ashing at high temperature in the presence of alkali, 3) dis-

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solution of the iodides from the ash by acids, 4) colorimetric determination of the reaction velocity between arsenious acid and ceric sulfate, a reaction catalyzed by the iodides present in the dissolved ash.

Performance

Blood sampling. — About 10 ml of blood is collected by venipuncture using a stainless steel needle. The sample is allowed to flow directly into a centrifuge tube. No syringe is used.

1) Precipitation and washing of the serum proteins. — 1 ml of serum is pipetted into a 50 ml Supremax tube[1] and mixed with 2.5 ml of zinc sulfate solution and 20 ml of double-distilled water. 3 ml of 0.75 N sodium hydroxide is added during stirring. The tube is allowed to stand for at least 3 hours and is then centrifuged for 15 minutes at about 800 g. The precipitate is resuspended with 10 ml double-distilled water and recentrifuged. This procedure is repeated twice.

2) Drying and incineration of the samples. — 2 ml of 4 N sodium carbonate is added to the washed precipitate and the tube is kept overnight at 92—95° C. For incineration the tube is placed in a preheated muffle furnace for 3 hours at 625° C.

3) Dissolving the iodides from the ash. — To the cooled incinerated sample is added 3 ml of double-distilled water, 2 ml of 2 N hydrochloric acid and 2 ml of 7 N sulfuric acid in this order.

4) Colorimetric determination of the iodide content. — 3 ml of the ash solution is mixed in a tube with 1 ml of arsenious acid solution. The tube is placed in a constant temperature bath maintained at 24 ± 0.1° C for about 30 minutes. Exactly 1 ml of ceric ammonium sulfate solution is added. Exactly 30 minutes later the light transmission of the solution is read against a reagent blank in a Beckman B spectrophotometer at 440 mμ. The amount of iodide present is determined with reference to a standard curve.

Blank. — One blank is carried through the entire procedure for each incineration. Furthermore each set of determinations is checked by parallel analysis of a "standard" serum. The blank values fairly constant and of the magnitude of 0.5—1 iodine per 100 ml. This slight variation o blank values is due chiefly to the varying a of iodine in different batches of hydrochloric

Reagents[1]

Cleaning. — The needles employed are car rinsed with double-distilled water and sterili a hot air oven at 140° C. The test tubes, as w all glassware, are placed in 5 per cent hydroc acid over night, rinsed off with distilled followed by double-distilled water and dri 110° C.

Distilled water. — All solutions should be pared in double-distilled water. Redistillation potassium permanganate should be done in a apparatus.

Zinc sulfate. — 125 g of $ZnSO_4 \cdot 7H_2O$ *analysi,* Merck, Darmstadt) and 125 ml 2.5 N H are made up to 1 liter with double-distilled

Sodium hydroxide. — 0.75 N (*pro analysi,* trokemiska AB, Bohus, Sweden). The soluti zinc sulfate and sodium hydroxide are bal until 5 ml of the acid zinc sulfate requires 6.8 ml of the alkali to turn phenolphthalein p nently pink.

Sodium carbonate. — 4 N (*pro analysi,* M Darmstadt).

Sulfuric acid. — 14 N and 7 N (double-di for research purposes, Merck, Darmstad).

Hydrochloric acid. — 2 N (at least 36.4 pe double-distilled, Merck, Darmstadt).

Arsenious acid. — 3.71 g of As_2O_3 (*pro a* Merck, Darmstadt) are dissolved in 50 N NaOH during stirring. 200 ml of water is and the solution neutralized with H_2SO_4 (req about 2.5 ml of the 14 N). Then 54 ml 14 N H_2SO_4 is added, and the solution ma to 500 ml. Iodine-free NaCl (*pro analysi,* M

[1] Supremax glass (Jena) is preferable to Pyrex glass because of its durability and higher melting point.

[1] All reagents used should be of highest obtainable. As we had great difficulties in f iodine-free reagents we thought it might be to give the makes of the reagents we em after testing a fair selection, but by no means those commercially available.

nstadt) is carefully dried in an oven at 110° C. g is dissolved in the 500 ml of reagent solution. eric ammonium sulfate. — 12 g of Ce $(SO_4)_2 \cdot$ $H_4)_2SO_4$ (iodine free, Hopkin & Williams, Ltd. lon) is added during stirring to 500 ml of N H_2SO_4. Occasional stirring will clarify the ally turbid solution within 30 minutes.

COMMENTS AND PRECAUTIONS

he discussion of the analytical procedure mited to the modifications of the original hod. Readers interested in a detailed ussion of the entire method are referred Barker (1948) and Barker *et al.* (1950, 1).

) Precipitation and washing of the serum eins. — When using zinc sulfate and um hydroxide in the amounts suggested Barker *et al.*, precipitation of the proteins occasionally incomplete. We therefore larger quantities of these reagents.

he test tube with the precipitate is ved to stand for at least 3 hours before rifugation, otherwise precipitation of the m proteins can be incomplete. Although urs is, as a rule, sufficient it is wise to with an 8-hour interval.

Drying and incineration of the samples. Contratry to the recommendation of Bar- *et al.*, who thoroughly mix the sodium onate and the precipitate, we place the um carbonate as a layer on top of the ipitate and do not stir it in. In our ion this gives more consistent results. true that bubble formation does occasion- occur but without any demonstrable loss dine material. On the contrary, the loss ng the later incineration is, if anything, than in the original method and is pos- ascribable to the use of larger amounts dium carbonate, zinc sulfate and sodium oxide.

We use and ordinary electric muffle furnace and maintain the temperature constant by means of a plugboard transformer for the regulation of the voltage to the furnace. The temperature is continuously recorded with an iron-constantan thermo-element. If an automatic electric furnace is used, it should preferably be provided with two heating systems; a powerful one for heating up the furnace and a less powerful one for maintaining the temperature of the heated furnace constant. The temperature is conveniently controlled by a "Fallbügel" regulator.

The ashing time and temperature should be kept as constant as possible. We have found incineration for 3 hours at $625 \pm 5°$ C suitable. It is impossible to avoid iodine losses during the incineration. The losses are of the order of 15 per cent. However, under the experimental conditions described the percentage lost is constant. It is thus possible to make corrections for the losses of iodine (see below). The variation in the recovery of iodine with the temperature and/or the duration of incineration is demonstrated in Table I. Any iodine still organi-

Table I. *Recovery of iodine from six different sera at varying temperature and duration of the incineration.*

Temperature °C	Duration in hours	Iodine recovered µg per 100 ml					
		No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
500	6	0.5	0.5	0.8	2.0	2.6	2.0
500	10	0.5	1.5	3.5	4.5	5.5	8.0
585	6	0.8	1.6	4.5	6.8	7.8	10.0
625	3	0.8	1.8	4.7	7.4	8.5	10.9
625	6	1.0	1.8	4.8	7.4	8.5	10.4

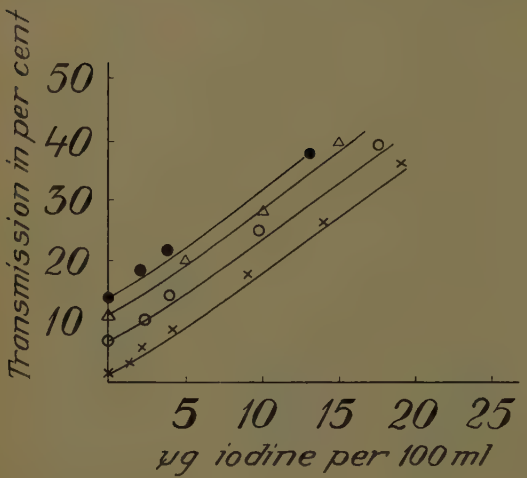

Fig. 1. Standard curves for four different sera showing the relationship between light transmission values and the iodine content of varying amounts of thyroxine added to serum.

cally bound after the ashing procedure will not catalyze the ceric ammonium sulfate reaction, and then protein-bound iodine recordings will be fictitiously low. Table I shows that combustion is incomplete both at 500° C and 585° C and that prolongation of combustion from 6 hours to 10 hours at 500° C increases the recovery, but the yield is still unsatisfactory. It is also demonstrated that at 625° C the losses of iodine do not increase when the ashing is prolonged from 3 hours to 6 hours. At temperatures above 650° C the losses of iodine will increase rapidly owing to volatilization.

3) Dissolving the iodides from the ash. — Contrary to the experience of Barker *et al.*, we have never observed any insoluble material with our procedure of incineration. The presence of insoluble material is probably due to incomplete combustion.

4) Colorimetric determination of the iod content. — The determination should made under carefully standardized conditi

The iodide content is read directly fro previously prepared standard curve. T curve was obtained in the following w To the precipitates obtained from 1 m serum from each of four subjects thyro was added in an amount of 0—0.4 µg. the samples were then subjected to the en procedure described above and the l transmission values were plotted against respective thyroxine-iodine concentrat (Fig. 1). The four curves are then shi horizontally to the right until the exten of the curves passes through the origi co-ordinate axes. The curves thus practi coincide and thereby form a compo standard curve (Fig. 2). This proce takes into account the varying amoun iodine originally present in the serum. transmission values were corrected for blank value which represents the light tr

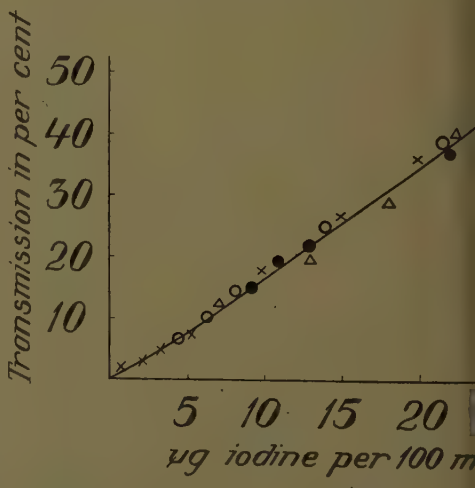

Fig. 2. Composite standard curve obtained fo four sera in Fig. 1.

able II. *Determinations of known unts of thyroxine and sodium iodide with reference to the standard curve.*

Substance	Iodine added μg	Iodine found μg
Thyroxine	.025	.030
	.050	.053
	.100	.101
	.150	.158
	.200	.203
Sodium iodide	.020	.025
	.050	.053
	.100	.110
	.150	.145
	.200	.183

on obtained from analysis of all reagents oyed.

ne correctness of the standard curve was ked against determinations on known unts of thyroxine and sodium iodide le II). The table demonstrates that the es obtained by using the standard curve ictitiously high for the smaller amounts yroxine and sodium iodide and tend to ictitiously low for larger amounts of oxine and sodium iodide. In the normal e of serum protein-bound iodine there ery good agreement between observed expected values.

eterminations were also made on dif- t sera to which sodium iodide had been d in varying amount (Table III). The recoveries argue for the correctness of tandard curve. The sodium iodide and oxine are added at the stage when m carbonate is added to the precipitate, before drying and incineration.

henever a new stock solution of cerium onium sulfate is prepared, the standard e must be checked. If satisfactory agree-

Table III. *Yield (endogenous + exogenous) of iodine from sera after addition of known amounts of sodium iodide.*

Serum iodine	Iodine added	Iodine recovered	
μg per 100 ml	μg per 100 ml	μg per 100 ml	per cent
No. 1 2.0	0	2.0	100
»	5	7.4	106
»	10	12.2	101
»	15	18.0	106
No. 2 5.7	0	5.7	100
»	5	10.9	102
»	10	15.3	98
»	15	21.2	102
No. 3 11.5	0	11.5	100
»	5	16.8	102
»	10	22.0	102
»	15	25.8	97

ment is not achieved, a new standard curve should be prepared. The standard curve can be constructed on the basis of analyses of different amounts of sodium iodide only, but it appears more reliable to base the standard curve on various amounts of mixtures of sodium iodide and serum.

The entire analysis can, if necessary, be carried out in two days. For routine work, however, it appears more suitable to do the analysis in three days, *i. e.* let the precipitate stand overnight, wash and dry on the next day, and incinerate and read the result on the third day.

ACCURACY OF THE METHOD

The accuracy of the method was determined on the basis of duplicate analyses of 100 consecutive sera. Each serum was analyzed on different days and with the use of two different furnaces. The standard error of a single observation was found to be 0.22 μg per 100 ml. The error appears to be

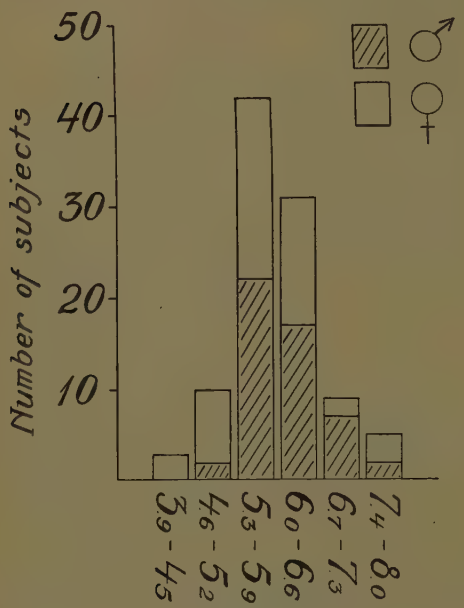

Fig. 3. Distribution of serum protein-bound iodine values in 100 apparently healthy subjects. The base of each column represents 1 σ (= 0.7 μg per 100 ml).

of the same order for both high and low values. However, the fairly high error in the percentages below 2 μg per 100 ml is of little practical significance, because this level is far below the lower normal limit.

NORMAL RANGE

The normal range (Fig. 3) was determined on the basis of samples from 100 registered blood donors in whom clinical examination had failed to show thyroid dysfunction. The mean value (M) was found to be 5.9 μg per 100 ml and the standard deviation of the mean (σ) 0.7 μg per 100 These figures did not vary with sex. V a range of M $\pm$ 3 σ, which includes 99.7 cent of all normal values, the normal limits be 3.8 μg per 100 ml and 8.0 μg per 100 Although there is a tendency to take M $\pm$ as the normal range for laboratory tests, wider range of M $\pm$ 3 σ appears to be n suited for the present method. In prac we have considered 4 to 8 μg per 100 m the normal range.

The value of the present method for diagnosis of thyroid disorders has been ported by Skanse (1955).

SUMMARY

A modification is described of Bar Humphrey & Soley's method for the de mination of serum protein-bound iodine. modification consists mainly of: the us larger quantities of zinc sulfate, soc hydroxide and sodium carbonate; dep tion of the sodium carbonate as a laye the top of the precipitate and not stirred and, in the colorimetric determination, use of a standard curve based on ana of known amounts of thyroxine or soc iodide added before incineration to p pitates from different sera.

The normal mean, as found for 100 ap ently healthy adults is 5.9 μg per 10 with a standard deviation of 0.7 μg 100 ml for either sex.

This modification has proved more s factory than the original method and itself to clinical work.

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THE CALCIUM AND PHOSPHORUS METABOLISM IN GASTRECTOMIZED PATIENTS*

By R. NICOLAYSEN and R. RAGÅRD

From the Institute for Nutritional Research, University of Oslo, Norway

(Received for publication June 11, 1955)

Gastrectomy in young dogs is followed by severe osteoporosis (Bussabarger, Freeman & Ivy, 1931) in adult age. The osteomalacia which follows steatorrhea is a well known entity.

The opportunity to study Ca and P metabolism in gastrectomized humans was offered and the work here presented was included in the study of the digestion in 15 gastrectomized patients, reported in the preceeding paper.

METHODS

The Ca and P intakes were measured accurately by analyses of food as eaten. Aliquots of the dried food samples and of urine and feces were wet ashed (nitric acid, perchloric acid mixture). The Ca and P analyses were made by the routine permanganate titration and colorimetric methods respectively.

RESULTS

The results are given in the table. They should be viewed on the background of the long term study of Ca metabolism in man conducted in this laboratory (Nicolaysen, Eeg-Larsen & Malm, 1953; Malm, Nicolaysen & Skjelkvale, 1955).

The following points are the essential ones. The Ca balance in man is subjected to a number of variations, such as abrupt variati from one fortnightly period to the follow and periodic variations over months. subject may be in a considerable nega balance for months, followed by a perio positive balance. The plurality of hum adapt well to low intakes (400—500 mg daily) by improved absorption. The urin Ca excretion is a personal constant years, some may excrete 100 mg while ot excrete 400 mg daily. In som the urinary is reduced when the intake is halved whe it remains constant in others.

Due to the fact that the present study cludes only one fortnightly period for person, only substantial deviations from values observed in normal persons shoul considered as representing a biolo reality.

However, the absorption of calcium clearly defective in a number of the pati the net absorption is frequently of a size negative value. The excess fat in the i stinal contents is probably responsible the reduced absorption. This may in be a direct effect of the excess fatty a However, in view of the fact that vitami is essential to Ca absorption also in a (Malm, Nicolaysen & Skjelkvale, 1955) min D deficiency may be partly respons

* Financial support received from New York Community Trust & Ely Lilly and Company.

*The daily Ca and P balances in gastrectomized patients.**

ient o.	Month after operation	Intake		Absorbed		Urine		Balance	
		Ca mg	P mg	Ca mg	P mg	Ca mg	P mg	Ca mg	P mg
10	3	438.0	776.8	— 53.5	507.4	77.9	515.0	—131.4	— 7.6
5	4	594.4	726.2	—111.8	413.3	97.1	557.3	—208.9	—144.0
6	4	519.0	647.4	14.1	339.7	63.9	505.5	— 49.8	—165.8
8	7	489.1	591.6	—225.0	197.3	216.3	857.8	—441.3	—664.1
7	8	449.8	522.8	—374.2	126.8	351.9	1004.0	—726.1	—877.2
6	9	467.5	840.7	28.0	557.7	169.5	652.1	—141.5	— 94.4
3	15	713.8	887.8	—132.9	520.8	208.7	813.0	—341.6	—292.2
4	15	476.9	628.9	— 86.2	321.2	77.5	638.5	—163.7	—317.3
2	16	865.8	1215.0	—202.3	433.5	186.6	669.6	—388.9	—236.1
2	20	778.4	877.2	32.4	601.4	79.7	761.3	— 47.3	—159.9
5	23	447.2	626.1	—438.5	215.4	73.0	796.4	—511.5	—581.0
1	25	693.6	963.5	127.1	679.9	81.7	732.4	+ 45.4	— 52.5
7	33	574.7	770.7	226.6	573.7	163.7	716.4	+ 62,9	—142.7
8	39	430.8	692.6	—235.1	396.4	48.7	681.1	—283.8	—284.7
9	43	291.3	560.9	— 25.8	310.8	56.8	516.8	— 82.6	—206,0

for other details see Nicolaysen & Ragaard (1955).

he urinary Ca in some of the patients was itely below the values found for normal ons. On the other hand relatively high ary Ca values were observed i some. The figure of over 300 mg in a man who t have been in a considerable negative Ca nce for many months is in line with rvations in normal men (Malm *et al.*,). It strengthens the contention that urinary Ca excretion is dominated by genous factors.

ne negative P balances observed may in be due to defective Ca absorption, in to the loss of body protein.

ne practical implication is clear. Such nts should receive liberal amounts of um and vitamin D. Such negative Ca nces as here reported cannot continue ears without serious consequences.

SUMMARY

The Ca and P balances were highly negative in a number of gastrectomized patients.

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MAXIMUM BREATHING CAPACITY AND VITAL CAPACI DETERMINED BY MEANS OF THE KROGH SPIROMET

By L. HAMRE and H. KJØRSTAD

From the Med. Dept., Halden komm. sykehus, Halden, Norway

(Received for publication June 6, 1955)

Evaluation of the pulmonary bellow function by measurement of the vital capacity only must be considered as rather unsatisfactory. Spirographic records of the maximum breathing capacity — the maximal ventilation which can be achieved by forced respiration, expressed in liters/min. — yields more adequate information about the bellow function as the time factor also is included.

Measurements of the maximum breathing capacity are being increasingly used in laboratory centres, but are not commonly used in hospitals. The reason is probably that the instruments used are often expensive and complicated.

Our aim has been to reconstruct an ordinary Krogh spirometer in such a way that the apparatus may be used for measurements of basal metabolism as well as for determinations of maximum breathing capacity and vital capacity.

There has been disagreement whether the maximum breathing capacity is a readily reproducible value. The majority are of the opinion that the values show a good reproducibility, whereas some maintain that a "learning effect" will be encountered in some subjects (Comroe, 1953).

Baldwin, Cournand & Richards (19 have published formulae for prediction maximum breathing capacity (based on and surface area) and vital capacity (b on age and height) in males and females

Maximum breathing capacity (liters/mi

Males: (86.5 — (0.522 × age in yea × surface area in sq. m.

Females: (71.3 — (0.474 × age in yea × surface area in sq. m.

Vital capacity (ml.):

Males: (27.63 — (0.112 × age in yea × height in cm.

Females: (21.78 —(0.101× age in yea × height in cm.

METHODS

Apparatus. (Fig. 1.)

An ordinary spirometer of the Krogh typ been used. A rapidly revolving recording is needed to get clear spirograms for regist the maximum breathing capacity. We have a cylinder, diameter 9 cm and height 14.5 cm connected it to the original drum, as show Fig. 1. In this way the speed of the kymogra trebled.

In order to determine what resistance the lime container and the valves represent, mea

1. The Krogh spirometer adapted for measure-
its of maximum breathing capacity and vital
acity. A new rapidly revolving drum (front
dle) is connected with the original drum (front
left) by a rubber strap.

its were made in 4 normal subjects. We found
the valves offered a considerable resistance,
that the values for the maximum breathing
acity for this reason became too low. Resistance
to the soda lime did not influence the values.
convenience we therefore chose to retain the
ainer but to substitute the valve box with a
ibe.

When the valves are removed, "dead space" in-
ses by a volume corresponding to the capacity
he tubings. This will cause an increase in the
ilation. Our apparatus can therefore not be
l for quantitative recording of the ventilation
rest. For measuring the maximum breathing
acity and the vital capacity the CO_2 accumula-
is not a disadvantage.

n the spirometer used by us the chart-speed was
mm/min. The volumetric calibration was
mm/liter.

Procedure

Normal values for maximum breathing capacity
vital capacity were determined in *50 subjects*
out cardiac or pulmonary disease, 25 males
and 25 females, distributed with 5 males and 5 females in each of the 10-year groups between 20 and 70 years. 10 males aged 25—43 were examined again 3 months after the first test.

All determinations were carried out in the standard way (Comroe) with the subjects sitting in a comfortable armchair.

Both vital capacity and maximum breathing capacity were measured 3 times with intervals of 5—10 minutes. The highest value of the three measurements was recorded as the final result.

As we carried out the tests under relatively constant exterior conditions we obstained from correcting the values according to room temperature and barometric pressure.

Fig. 2 shows a normal spirogram with readings. It is not possible to read the vital capacity from the tracings with a greater accuracy than 50 ml, and an accuracy beyond whole liters for the maximum breathing capacity is illusory.

RESULTS

Table I presents our normal material. It is tabulated in the same form as the material of Baldwin *et al.,* and the P-values stated refer to statistical comparisons between the two materials.

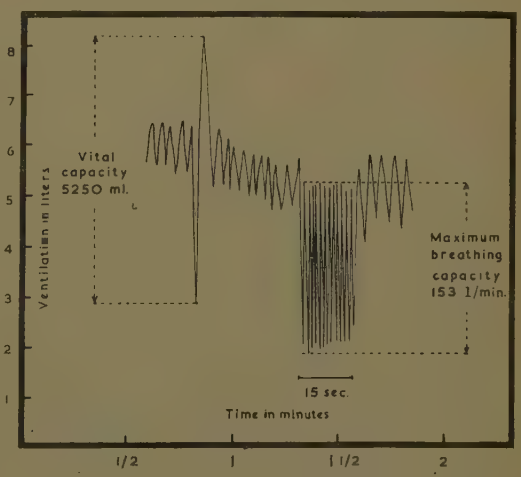

Fig. 2. A normal spirogram.

Table I. *Physical characteristics, vital capacity and maximum breathing capacity 25 healthy men and 25 healthy women.*

(P: Statistical comparison with normal groups (Baldwin, Cournand and Richards, 1948.))

	Group I: 20—34 years				Group II: 35—49 years				Group III: 50—70 ye			
	No.	Mean	S.D.	P Probability	No.	Mean	S.D.	P	No.	Mean	S.D.	
Men												
Age, years	10	29.3	2.4	0.02	5	44.2	4.09	0.4	10	59.9	6.3	0.9
Height, cm	10	176.2	5.5	0.3	5	176.0	6.75	0.2	10	173.3	8.7	0.1
Weight, kg	10	71.7	10.6	0.1	5	81.0	13.1	0.02	10	76.8	10.5	0.01
Body surf., sq.m.	10	1.86	0.11	0.05*	5	1.96	0.15	0.05*	10	1.90	0.18	0.1
Vital cap. in ml.	10	5135	751	0.001***	5	4590	444	0.1	10	4355	926	0.01
Max. breathing cap. in l/min.	10	146.8	10.9	0.01**	5	117.4	20.4	0.4	10	109.2	25.0	0.02
Women												
Age, years	7	25.1	4.5	0.9	8	42.5	3.7	0.6	10	58.7	5.7	0.7
Height, cm	7	165.3	6.5	0.2	8	163.8	4.4	0.9	10	166.0	3.8	0.00
Weight, kg	7	62.1	5.1	0.3	8	65.5	5.1	0.5	10	68.0	9.6	0.8
Body surf., sq.m.	7	1.53	0.09	0.3	8	1.71	0.02	0.001***	10	1.74	0.12	0.4
Vital cap. in ml.	7	3600	537	0.05*	8	3681	503	0.001***	10	2880	466	0.02
Max. breathing cap. in l/min.	7	92.4	10.7	0.7	8	89.0	14.5	0.9	10	76.9	15.3	0.6

P = Probability. The value refers to data given in t-table (Bonnier & Tedin, p. 322).
*, **, ***. The asterisks indicate the probability-levels and refer to tables for P.

* = P: 0.05 (Barely significant).
** = P: 0.01 (Significant).
*** = P: 0.001 (Highly significant).

Maximum breathing capacity

A mean value of 126 liters/min. with a standard deviation of 25.7 was found for all males in our normal material. The mean value for the females was 85 liters/min. with a standard deviation of 15.1.

Table I shows no significant difference between our values and those of Baldwin *et al.* except in the youngest age group of males, in which our values are higher. This may be explained by the fact that in this group our normal subjects differ from those of Baldwin *et al.* with regard to age and surface area.

Table II shows a statistic comparison of the recorded values in our material with the

Table II. *Observed and predicted v of maximum breathing capacity (MBC 25 healthy men and 25 healthy wom*

	Mean L./Min.	S.D.
Men		
Observed MBC	126	25.7
Predicted MBC	120	17.8
Observed MBC in per cent of predicted	105	14.8
Women		
Observed MBC	85	15.1
Predicted MBC	86	11.3
Observed MBC in per cent of predicted	99	14.4

es for the same material computed rding to the formulae of Baldwin *et al.* he maximum breathing capacity. There significant difference between measured predicted values.

of our normal subjects were re- ined 3 months after the first test. The n value at the first test was 145.9 liters/ with a standard deviation of 11.2 — at second test 146.8 liters/min. with a dard deviation of 11.7. For single ects the greatest decrease in value was ters/min., the greatest increase 9 liters/ There was no significant variation be- n measurements in the 10 subjects as ole (P greater than 0.9), *i.e.* no evidence "learning effect".

Vital capacity

n average value of 4714 ml with a dard deviation of 835 was found for all s in our material. For the females the average value was 3338 ml with a standard deviation of 611.

From table I it appears that the vital capacity in our material is on the whole significantly higher than in the material of Baldwin *et al.*, whereas, with a single exeption (height in the female group 50—70 years), there is no difference between the materials as regards height and age.

Table III shows the result of a statistic comparison between the recorded values for our material and the predicted values of vital capacity by the formulae of Baldwin *et al.* The registered values are significantly higher than the predicted ones both for males and females.

b l e III. *Observed and predicted values vital capacity (VC) in 25 healthy men and 25 healthy women.*

	Mean ml.	S.D.	P
en			
rved VC	4714	835	0.001***
cted VC	3980	363	
rved VC in cent of predicted	118	14.5	
men			
rved VC	3338	611	0.001***
cted VC	2874	261	
rved VC in cent of predicted	116	17.1	

DISCUSSION

The supplementary equipment described above has, for our apparatus, been supplied by a tinsmith, and the cost amounted to 20 Norwegian crowns.

The apparatus can easily be changed from a spirometer into an apparatus for metabolic measurements. In our department metabolic determinations and spirometric tests are carried out daily with the same apparatus.

We believe the Krogh spirometer in the described, modified form serviceable for measurements of the *maximum breathing capacity* — a test which is now being increasingly used for evaluation of pulmonary insufficiency. Georg (1952) has previously used the Krogh spirometer for measurements of the maximum breathing capacity, but found too low values. Our modifications of the apparatus seem to cancel this objection. The concordance between our measured normal values and the predicted values by the classic formulae of Baldwin *et al.* seems

to offer a special advantage. The values have shown a good reproducibility in a minor control material.

The *vital capacity* values are in our normal material higher than those of Baldwin *et al.*, which is in concordance with the findings of other authors, among them Larmi (1954).

The Krogh spirometer is the apparatus most frequently employed in Scandinavia for measuring basal metabolism. For laboratories without special equipment it seems to be well adapted for measuring the maximum breathing capacity and the vital capacity.

SUMMARY

The Krogh spirometer with a simple supplementary equipment has been employed to measure maximum breathing capacity and vital capacity. The apparatus is described. The values for a normal material of 25 males and 25 females are presented and compared with the normal material of Baldwin, Cournand & Richards (1948). The values fo maximum breathing capacity have sho good reproducibility. The advantage o simple equipment is pointed out. It is fi stated that the apparatus is well suit measurements of maximum breathing city and vital capacity.

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LORIMETRIC DETERMINATION OF FRUCTOSE WITH INDOL

By M. J. KARVONEN and M. MALM

om the Institute of Occupational Health, Helsinki, Finland, and from the Department of Metabolic Research, Wenner-Gren's Institute, University of Stockholm, Sweden

(Received for publication July 30, 1955)

ructose may be quantitatively determined he presence of other reducing sugars r separation with paper chromatography by using color reactions more or less ific for fructose. With the most selective hese, like Cole's modification of Roe' 34) resorcinol method, or with that of ashi & Peters (1950), fructose gives a r approximately 100 times as intense as equal amount of glucose (Gray, 1950; erman & Villee, 1954). The diphenyl- ne method (van Creveld, 1927; Herbert, 3; Corcoran & Page, 1939; Kerdrick, 2) is less selective between glucose and tose, and may give a color also with r nonfructose compounds (Hagerman & ee).

hough the present methods for fructose satisfactory in many respects, a need may sionally arise for more selective or more itive methods of determination. A sen- e color reaction with skatole has been ied by Jordan & Pryde (1938), and equently used Pryde (1946). The writers ne present study decided to test whether lor reaction given by indol with fructose n acid solution (Jordan & Pryde) could sed with advantage for the determination ructose. The study resulted in a simple hod for fructose; the new method is more than 200 times as sensitive for fructose as for glucose, and approximately twice as sensitive for fructose as Cole's resorcinol method.

METHOD

Indol gives an intense yellow-orange color with small amounts of fructose in the presence of concentrated HCl, while *e. g.* tryptophan gives a red, less intense color in the same circumstances. Indol is only slightly soluble in water at room temperature. Therefore, it was first used as a solution containing 25 mg in 100 ml ethanol. Ethanol caused a considerable formation of color also in the absence of fructose, and the use of an aqueous reagent was therefore found preferable. The reagent was made by dissolving 25 mg of indol in 100 ml 0.2 per cent benzoic acid (to prevent microbe growth) and filtering it before use.

As an *acid,* both sulphuric and hydrochloric acids were tested. After adding sulphuric acid, the temperature of the mixture rises, and the heat of mixing causes a relatively fast development of color also with glucose. Therefore, hydrochloric acid only was used. If the concentration of hydrochloric acid in the reaction mixture is reduced, the mixture turns red; the yellow-orange color resulting from the use of more concentrated

HCl is markedly more intense. The use of 10 volumes of concentrated HCl, of one volume of the sample, and one volume of the indol reagent was found satisfactory for the development of the yellow-orange color.

The *extinction* of the color at various wavelength is shown in Fig. 1. The maximum is at 470 $\mu\mu$, which is therefore used for the readings. The extinction was found to increase as a practically linear function of the concentration up to 10 mg per cent; at higher concentrations the extinction rises slightly less than is to be expected from linearity.

According to Jordan & Pryde, ketohexoses only among a large group of substances with aldehyde and ketone groups give a fast color reaction with compounds having the indol nucleus. Thus, the ketohexose *sorbose* was found to give the same reaction; the extinction of 1 mg per cent sorbose was 60 per cent of that given by a similar concentration of fructose.

On *heating* the reaction mixture, 1 mg per cent fructose initially gives a color stronger

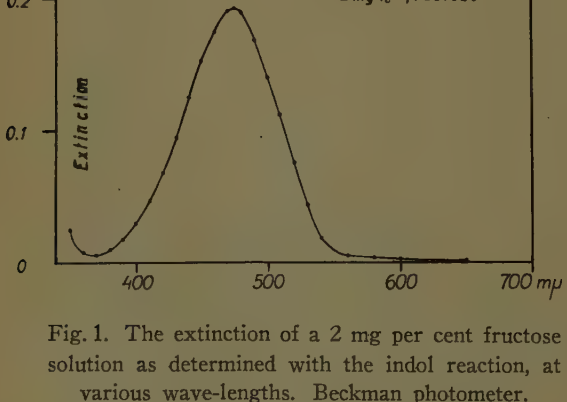

Fig. 1. The extinction of a 2 mg per cent fructose solution as determined with the indol reaction, at various wave-lengths. Beckman photometer.

Table I. *Extinction of mixtures of 0.5 indol reagent + 0.5 ml sugar solution + 5 concentrated HCl, heated for varying ti at + 50° C. The last column indicates h many times more sentitive the reaction is fructose than for glucose.*

Time min.	100 mg% glucose	1 mg% fructose	Ext. 1 mg % fructose / Ext. 100 mg% glucose
10	0.024	0.0515	215
20	0.062	0.084	135
30	0.087	0.1005	115
40	0.118	0.110	93
60	0.158	0.124	79

than 100 mg per cent glucose, but if hea of the mixture is continued, the color creases with glucose, after the reaction fructose is completed (Table I). Thus, ideal conditions for the determinatio fructose are such that the reaction with f tose is practically completed, whereas with glucose still is minimal. After te a series of *temperatures,* 50° C was ch as most suitable, where the color devel rapidly enough, but not too fast to es control.

The time of heating required v slightly according to the volumes and type of glassware used. When the volume is 6 ml and ordinary test tubes used, 20 min. has proved practical. Wi total volume of 30 ml and correspondi larger tubes, 30 min. is appropriate. Ta shows a comparison of the extinction v of some solutions of fructose and glucos they vary with the duration of the hea

For samples of blood, *deproteinization* sodium hydroxide and zinc sulphate (S gyi, 1930) or cadmium sulphate (Fuji

ke, 1931) have been used. Fructose l to blood is recovered quantitatively the indol method.

ndard procedure. The following was chosen standard procedure for protein-free samples ning at least 0.5 mg per cent fructose.

0.5 ml of the sample and 0.5 ml of the indol reagent are mixed in a test tube.

5 ml of concentrated HCl is added, and the tube is immediately placed in a water bath at + 50° C.

After 20 min., the tube is cooled in running water, and the color is read at 470 $\mu\mu$. When the Coleman photometer was used, 10 mm cuvettes were found suitable.

he fructose concentration is less than 0.5 mg nt, the use of greater optic depths is advis- The authors have in such a case used 5 times volumes and a cuvette with an light path mm.

e results are calculated by comparison with rds in the same batch, of the same concentration range, in the usual way. If glucose or other aldohexoses are present, their concentration has to be determined independently, and the extinction due to the non-fructose compounds has to be subtracted from the found values. This correction assumes importance when the concentration of glucose is more than 100 times that of fructose. An eventual contribution of non-fermentable chromogens — like sorbose — to the color may be checked by making the indol determination after a preceding removal of glucose and fructose by incubating the sample for 4 hrs at room temperature with washed baker's yeast.

The indol reaction may also be used for the determination of glucose, by heating the reaction mixture at higher temperature, until the development of color is completed.

SUMMARY

A simple method for the colorimetric determination of ketohexoses with indol and concentrated HCl is described.

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THE NITROGEN CLEARANCE OF THE LUNGS

By J. GEORG

From Medical Dept. B, Rigshospitalet, Copenhagen, Denmark

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At the beginning of the present century it was generally assumed that alveolar air is of uniform composition throughout the lungs, and that different parts of the lungs participate equally in the ventilation. Doubt as to the correctness of this opinion was first expressed by Keith (1908, 1909), who from anatomical considerations suggested that the basal and ventral parts of the lungs were better ventilated than the apical and dorsal. A few years later Sonne (1915), working with the nitrous oxide method for estimation of the minute volume of the heart, published a series of experiments which he interpreted as giving evidence of the unequal composition of alveolar air. He stated as his opinion: "It can hardly be taken as proved that alveolar air under normal circumstances is of homogenous composition, or that it ever may be rendered homogenous". In later experiments he obtained further evidence supporting this assumption (Sonne, 1916, 1918—19). Krogh & Lindhard (1917) during their determinations of dead space also obtained some evidence of the incomplete mixing of gases in the lungs, and Haldane, Meakins & Priestley (1918—19) studying the effort syndrome reached a similar opinion.

The investigations initiated by these kers have been followed in later years large series of experiments which o whole seem to support the view that al air cannot be considered as a comp homogenous mixture. A detailed revi earlier experiments was given by Rau (1946), and the whole subject has re been reviewed by Fowler (1952).

As the inequalities of air distributio markedly accentuated by some pathol alterations of the lungs, an estimation degree of uneven distribution has been of value in the assessment of pulmonary tion. Two main lines have been follow the design of experiments: Analys alveolar air from a single breath, or an of a series of respirations destined eit obtain equilibrium of mixture in a system or to follow the washout of an cator gas in an open system.

Analysis of a single breath was firs formed by Nielsen & Sonne (1932), used an apparatus specially construct obtain fractionated samples of alveola The method was used further by (1934, 1936). Roelsen, using inhalati hydrogen, developed the test into a although rather laborious pulmonary fu test (Roelsen, 1934, 1937, 1938, 193 was applied to silicotic subjects by R

[1] The work was supported by a grant from The Danish State Research Foundation.

ay (1940) and to patients with pulmo- tuberculosis by Gad (1943). Recently ilar test has been introduced by Fowler 9) and Comroe & Fowler (1951), who continuous analysis of the nitrogen con- ration of the expired air.

rath (1944) employed the second method vestigation. He studied the equilibrium ined during quiet breathing in a closed em, using hydrogen as indicator gas, and pared his findings with the results theo- ally predicted on the hypothesis of per- mixing. Bates & Christie (1950) studied mixture of helium in a closed system, expressed their results as an index of ng efficiency derived from the ratio of retically predicted / actual number of irations necessary to obtain 90 per cent librium. A similar procedure was used Briscoe, Becklake & Rose (1951) and coe (1952), who used the curves to nate a well ventilated and a poorly ven- ed part of the functional residual capa- Nitrogen was used as indicator gas by fe & Carlson (1950), who also studied librium in a closed system.

he open-circuit method for measuring ual volume, introduced by Darling, Cour- l & Richards (1940), gave rise to an- r way of estimating intrapulmonary air ibution. It was found (Cournand, Bald- Darling & Richards, 1941) that samples veolar air taken after seven minutes of gen breathing varied considerably in gen content. Whereas normal subjects values below 2.5 per cent, many patients specially emphysematous subjects — had ch higher nitrogen content. A further sis of the nitrogen elimination during en breathing was carried out by Darling, Cournand & Richards (1944). Other studies of the nitrogen washout process have been made by Bateman (1946), Bateman, Boothby & Helmholz (1949), Bateman (1950—51), Becklake (1951, 1952), Boothby, Lundin & Helmholz (1948), Colldahl & Lundin (1952), Fowler, Cornish & Kety (1952) and Robertson, Siri & Jones (1950). The last two papers contain a thorough discussion of the theoretical foundations for the clearance process.

The results of these tests have been expressed in different ways by different workers. The measurement used most has been the one labelled by Cournand *et al.* (1941) "pulmonary emptying rate", *i. e.* the alveolar nitrogen percentage after seven minutes of quiet breathing in oxygen. The test has the advantage of being simple and practical, but the disadvantage of fixing only a single point on a rather complicated curve.

The elimination of nitrogen during oxygen breathing will be dependent on the alveolar ventilation (*i. e.* on tidal volume, dead space and respiratory rate of the subject) and on intrapulmonary air distribution. In an ideal lung with perfect mixing and a constant ratio of tidal volume/functional residual capacity, the decrease in alveolar nitrogen concentration will follow a simple exponential function:

$$F_{A_{N_2}^n} = \left(\frac{FRC}{FRC + (V_T - V_D)}\right)^n \times F_{A_{N_2}^o}$$

where:

$F_{A_{N_2}^o}$ = alveolar nitrogen concentration at zero time.

$F_{A_{N_2}^n}$ = alveolar nitrogen concentration after n respirations.

FRC = functional residual capacity.

$V_T - V_D$ = tidal volume — dead space
= effective tidal volume.

If FRC, V_T and V_D are kept constant the equation reads

$$F_{A_{N_2}^n} = k^n \times F_{A_{N_2}^0}$$

which signifies that alveolar nitrogen percentage plotted in a semilog system against the the number of respirations will produce a straightline graph, the ordinate at zero being the initial alveolar nitrogen concentration, and the slope depending on the ratio of FRC to (FRC + effective tidal volume), *i. e.* the alveolar dilution ratio.

If the respiratory frequency and tidal volume are kept constant, a straightline relationship will, of course, also be obtained if log alveolar nitrogen percentage is plotted against time or against respiratory minute volume.

In actual experiments on human beings the curves obtained will deviate from the ideal curve because the alveolar dilution ratio is not absolutely constant, because nitrogen is eliminated from the body when pulmonary nitrogen concentration decreases, and because the intrapulmonary gas mixing is not perfect. It has been shown that young normal subjects produce curves which are reasonably near to those theoretically predicted. Elderly normal subjects, as will be shown in the present study, often deviate considerably; and patients with pulmonary disease may produce curves which are very far from the theoretical straightline relationship. These deviations are presumed mainly to be caused by uneven intrapulmonary gas distribution.

As the pulmonary emptying rate of Courand *et al.* is at present the most frequently used test for the estimation of intrapu nary mixing, the studies presented here aimed at an evaluation of this test. T fore 2.5 per cent alveolar nitrogen co tration has been adopted as an end p the time taken by normal subjects an patients with pulmonary disease to this point has been noted, and the ventil has been recorded during the proce From these measurements are calculate total ventilation needed by the subje empty his lungs down to 2.5 per cent alv nitrogen concentration. This measure is suggested as a useful estimation o degree of uneven ventilation in the lun

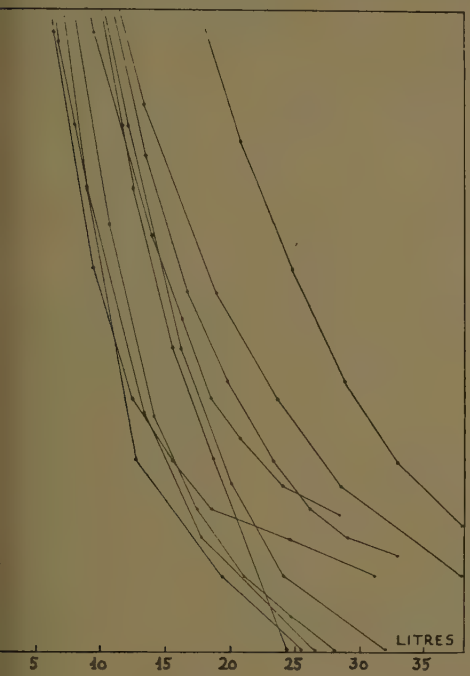
MATERIAL AND METHODS

The investigations were carried out on a of normal subjects and on patients with v pulmonary diseases.

The normal series consisted of 50 subjects, ages ranged from 12 to 70 years. Some of were completely normal persons (physicians, ratory technicians), but most of them we patients without cardiorespiratory complaint with no demonstrable heart or lung disease

The series of patients with pulmonary di comprised 50 subjects with emphysema lungs, 50 subjects with bronchial asthma or c bronchitis (without emphysema), and 50 s with silicosis. The subjects included emphysema group were patients with a diagnosis of emphysema, which could be con by the following results of the pulmonary fu tests: Total lung volume inside or highe predicted normal range; residual capacity 40 per cent of total lung volume; and vent capacity definitely reduced (*viz.* maximum ing capacity below 50 per cent of predicted and timed vital capacity (1 second value) 60 per cent). (The normal standards were from Needham, Rogan & McDonald (1954)

The experiments were performed with the sitting in a deck chair. Before the start



1. Alveolar nitrogen concentration during
gen breathing in 10 young, normal subjects
ler 30 years). Ordinate: Alveolar nitrogen per-
age. Abscissa: Total ventilation of the subject,
sured from the beginning of oxygen breathing.

eriment a rest period of some 10 minutes was
wed. Following this the subject was connected
mouthpiece and valve, breathing room air
ugh corrugated rubber tubing. When the
thing was judged to be quiet and stable the
ect was switched to inhalation of pure oxygen,
the expiratory air was measured by passing it
ugh a wet gasmeter. During the experiment a
tion of the expiratory air was continually
ed out through a needle valve, placed immedi-
behind the expiratory valve, and was ana-
d in the nitrogen meter (Lilly & Anderson,
4), as modified by Lundin (1952)). Readings
taken at intervals of 15—30 seconds; the
ilation of the subject was measured for at
5 minutes; and the respiratory rate was
ted for one minute at the beginning and to-
ls the end of the experiment.

The data presented are the respiratory rate, the minute volume of respiration (ambient temperature, pressure, saturated with water — the room temperature at the investigations being between 20° and 23° C), the time needed to bring the alveolar nitrogen concentration to 2.5 per cent, and the total ventilation used by the subject to produce this effect.

RESULTS

Figs. 1 and 2 show the recorded values for alveolar nitrogen concentration during oxygen breathing plotted against the total ventilation used by the subject, for 10 young and 10 elderly subjects. It is seen that the curves obtained with the young subjects (Fig. 1) come reasonably near the theoretically expected straight line — a deflection of

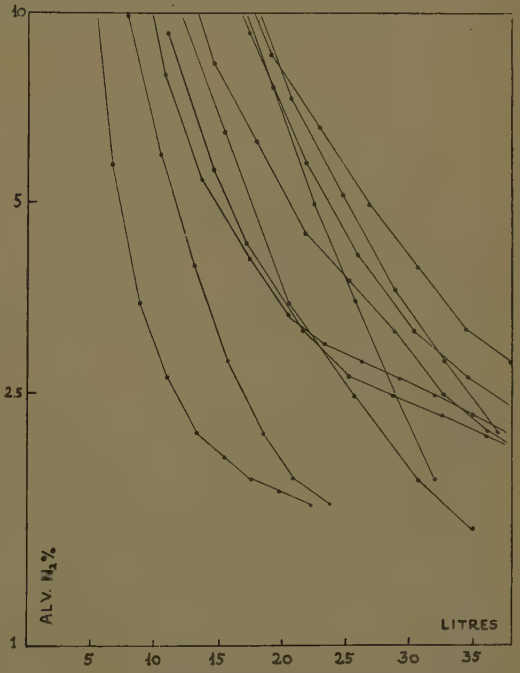


Fig. 2. Alveolar nitrogen concentration during oxygen breathing in 10 elderly, normal subjects (over 50 years). Coordinates as for Fig. 1.

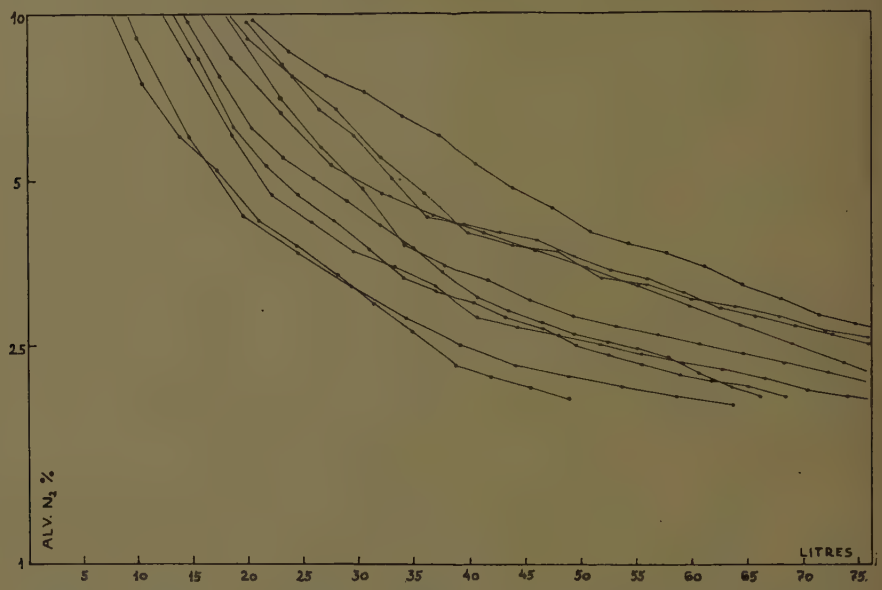


Fig. 3. Alveolar nitrogen concentration during oxygen breathing in 10 emphysematous subj
Coordinates as for Fig. 1.

the last part of the curve being inevitable on account of the nitrogen elimination from the body. It will be seen further that the 2.5 per vent value chosen by Cournand *et al.* is not unreasonable, as the deflection of the curve usually occurs below this point. (If a single point is to represent such a curve, the most useful will be the lowest point on the straight part of the curve).

In elderly subjects, however, the picture has already changed (Fig. 2). The curves are more dispersed and the deflections occur earlier in many cases. In others the curves are very similar to those produced by young subjects.

A further change is seen in the curves when emphysematous subjects are examined (*Fig. 3*). The slopes are significantly altered and the straightline relationship between log

Table I. *Normal subjects 10—30 y*

	Sex	Ventilation litres /min.	Rate /min.	Minutes needed to reach 2.5 %alv. N_2	T v til
G.B.	F	6.6	14	2.50	
A.B.	F	7.0	21	2.00	
H.W.	M	6.8	12	3.75	
T.J.	F	7.0	16	2.25	
T.N.	F	6.2	13	2.00	
M.N.	M	9.4	16	3.00	
M.S.	M	7.4	17	2.50	
K.T.	F	6.6	15	3.25	
R.H.	F	5.4	17	3.25	
S.G.	M	8.0	13	2.25	
B.A.	M	8.4	13	2.75	
H.H.	F	7.6	15	3.50	
K.M.	M	6.2	21	2.00	
G.S.	F	8.8	16	1.50	
B.R.	M	7.8	20	2.25	
E.F.	F	6.8	20	2.50	
D.S.	M	5.6	12	3.00	
Mean		7.2	16	2.6	

ble II. *Normal subjects 30—50 years.*

	Sex	Ventilation litres /min.	Rate /min.	Minutes needed to reach 2.5% alv. N_2	Total ventilation
	M	8.2	12	4.00	33
	M	5.8	13	5.00	29
	M	6.0	13	4.50	27
	M	6.2	14	2.75	17
	M	6.8	16	4.50	31
	M	7.4	15	4.00	30
	F	8.0	18	3.00	24
.	M	5.0	15	3.00	15
	F	4.8	10	4.25	20
.	M	8.2	9	3.75	31
	F	8.0	22	2.75	22
.	M	7.2	17	4.25	31
	F	8.2	16	1.75	14
.	F	8.0	21	3.50	28
.	M	7.0	12	3.25	23
	F	7.2	19	3.50	25
	M	5.8	14	5.00	29
Iean		6.9	15	3.7	25.2

ble III. *Normal subjects 50—70 years.*

	Sex	Ventilation litres /min.	Rate /min.	Minutes needed to reach 2.5% alv. N_2	Total ventilation
.	F	4.4	14	2.75	12
	M	7.8	11	3.50	27
.	M	5.8	10	5.25	30
	M	6.6	14	2.75	18
	F	4.4	13	4.00	18
.	M	10.2	17	2.50	25
.	M	6.6	14	3.75	25
.	M	7.6	10	5.50	42
.	M	8.6	13	4.25	37
	M	9.4	15	4.75	45
	M	6.4	12	4.50	29
	F	5.2	16	3.25	17
	F	8.0	15	4.25	34
	M	8.2	18	4.25	35
.	M	7.2	13	4.50	32
	M	7.2	13	4.00	29
ean		7.1	14	4.0	28.4

Table IV. *Average values for nitrogen clearance in 50 normal subjects.*

	Mean	SD	Observed Range
Ventilation (Litres per minute, ATPS)	7.1	1.3	4.4—10.2
Respiratory rate	15	3	9 —22
Minutes needed to reach 2.5% alveolar N_2	3.4	1.0	1.5— 5.5
Total ventilation needed to reach 2.5% alveolar N_2 (Litres, ATPS)	25.0	7.8	12 —45

alveolar nitrogen percentage and ventilation, which signifies an even intrapulmonary gas distribution, has disappeared. Such curves may be treated as representing a combination of several differently ventilated spaces — some well ventilated, other more poorly (Robertson, Siri & Jones, 1952).

More detailed data from the measurements on normal subjects, separated into three age groups, are given in Tables I, II and III. It is seen that the respiratory minute volume and frequency are almost identical in these groups, whereas the time and consequently the total ventilation needed to reduce the alveolar nitrogen concentration to 2.5 per cent on the whole increases with increasing age. As the functional residual capacity remains the same in different age groups (Needham, Rogan & McDonald, 1954) this must be taken to imply an increasingly uneven distribution of inspired air with advancing age.

The mean values for the normal subjects as a whole are given in Table IV. The measurements obtained in three groups of patients

Table V. *Average values for nitrogen clearance in 50 emphysematous subjects.*

	Mean	SD	Observed Range
Ventilation (Litres per minute, ATPS)	7.9	1.5	5.4—12.6
Respiratory Rate	18	4	11—28
Minutes needed to reach 2.5% alveolar N_2	7.8	2.8	4.0—20.0
Total ventilation needed to reach 2.5% alveolar N_2 (Litres, ATPS)	60.2	19.9	33—136

Table VI. *Average values for nitr clearance in 50 patients with chronic b chitis and bronchial asthma withou emphysema.*

	Mean	SD	Obse Ra
Ventilation (Litres per minute, ATPS)	7.7	1.7	4.6–
Respiratory Rate	16	3	10–
Minutes needed to reach 2.5% alveolar N_2	4.4	1.7	1.25
Total ventilation needed to reach 2.5% alveolar N_2 (Litres, ATPS)	32.5	13.2	12–

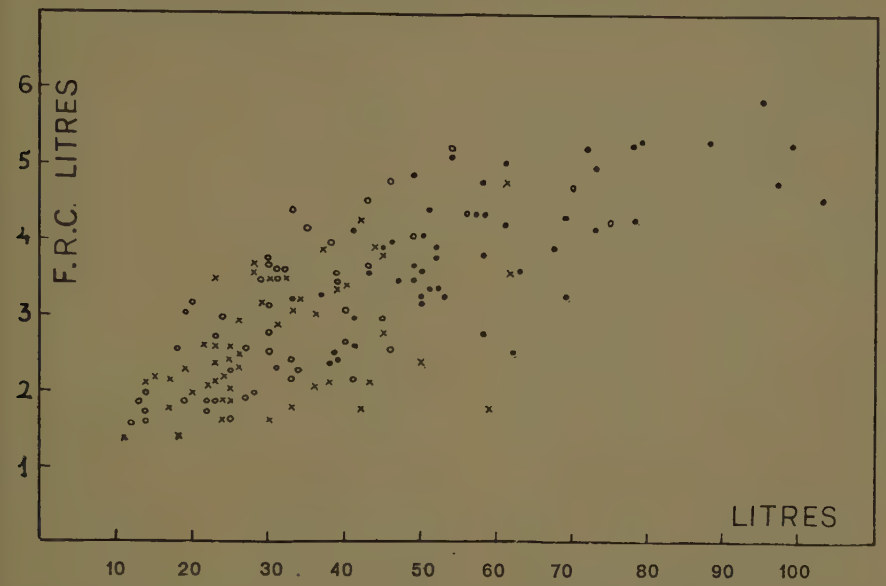
Table VII. *Average values for nitr clearance in 50 patients with silicos*

	Mean	SD	Obse Ra
Ventilation (Litres per minute, ATPS)	7.7	1.5	5.4–
Respiratory Rate	18	4	11–
Minutes needed to reach 2.5% alveolar N_2	4.1	1.5	1.50
Total ventilation needed to reach 2.5% alveolar N_2 (Litres, ATPS)	30.9	11.7	11–

with pulmonary disease are presented in Tables V, VI and VII. A distinction has been kept between emphysematous subjects and asthmatic or bronchitic subjects without emphysema, whereas the group of silicotic subjects contains some which may be considered as having emphysema as well.

The patients generally have somewhat higher ventilation than the normal subjects, and a similar, although more pronounced, difference is noted regarding the respiratory frequency. Notwithstanding this tendency to higher ventilation and more frequent respiration, the patients take longer to empty their lungs down to 2.5 per cent alveolar nitrogen concentration. The intergroup difference in this respect is highly significant as between the emphysematous and the normal subjects (4.4 minutes $\pm$ 0.4), less pronounced between the two other groups and the normal subjects (1.0 $\pm$ 0.3 and 0.7 $\pm$ 0.3 respectively).

The nitrogen washout volume (*i. e.* the total ventilation required to reduce the alveolar nitrogen percentage to 2.5) demonstrates these differences more markedly. In this respect the difference between emphysematous and normal subjects am to 36.3 $\pm$ 3.0 litres; in the case of the chitic subjects without emphysema, 8.5 litres, and in the case of the silicotic sub 6.9 $\pm$ 2.2 litres. The emphysematous jects stand out from all the other gr whereas any difference between the sil subjects and the bronchitic subjects wi



g. 4. The nitrogen washout volume (*i. e.* the total ventilation used by the subject from the beginning of oxygen breathing until the alveolar nitrogen concentration has reached 2.5 per cent) plotted against functional residual capacity.
o subjects with bronchitis or bronchial asthma, without emphysema.
× silicotic subjects. • emphysematous subjects.

physema is probably fortuitous (1.6 ± 2.5 es).

Fig. 4 shows the nitrogen washout volume tted against the functional residual volume he subject. Some correlation is evident, also a fairly wide "scatter" among different subjects with the same functional residual capacity *(e. g.* a clearance volume between 33 and 103 litres for functional residual capacity of about 4.5 litres).

f 45 litres is chosen as the upper normal t for the nitrogen washout volume, then of 50 emphysematous subjects (82 per t) are deemed abnormal by this test, reas 7 (14 per cent) of the subjects with nchitis *etc.*, but without emphysema, fall side the normal limits, and only 4 (8 per t) of the silicotic subjects do so.

COMMENTS

The criteria laid down by Cournand *et al.* (1941) for the estimation of pulmonary emptying rate, *i. e.* alveolar nitrogen concentration below 2.5 per cent after 7 minutes of oxygen breathing, have been fullfilled by each of the 50 normal subjects tested. In the present series the longest time taken by any normal subject to empty his lungs down to 2.5 per cent nitrogen was 5.5 minutes. The difference between the normal values of Cournand *et al.* and those of the present series may be partly due to the differences in the method used for obtaining alveolar air. Cournand *et al.* employed the Haldane-Priestley technique and took their samples from the last part of a deep expiration, in the present study the highest nitrogen concen-

tration recorded during a normal expiration has been employed.

As the time necessary to produce a certain reduction in pulmonary nitrogen concentration varies — other things being equal — with the ventilation of the subject, a better delimitation of normality may be obtained by taking this into account. The hyperventilation often demonstrated by patients with pulmonary disease tends to obscure the difference between normal and pathologic subjects as regards the pulmonary emptying rate.

In the present series of normal subjects the total ventilation needed to reduce the alveolar nitrogen concentration to 2.5 per cent was on the average around 25 litres, with 45 litres as the upper limit. It may be found profitable to modify the Cournand test by taking ventilation and not time as the limiting factor. This may be done simply by leading the expiratory air through a gas meter, and taking the final alveolar sample not after 7 minutes as originally proposed, but after a certain volume of air has been expired, *e. g.* 45 litres according to the present findings.

In the 150 patients with pulmonary disease an alveolar nitrogen percentage higher than 2.5 per cent after 7 minutes of oxygen breathing was found in 26 (52 per cent) of the emphysematous subjects, in 4 (8 per cent) of patients with bronchitis or bronchial asthma without emphysema, and in 3 (6 per cent) of patients with silicosis. A pathological nitrogen washout volume — according to the criteria outlined above — was found, however, in 82 per cent of the emphysematous, 14 per cent of the bronchitic, and 8 per cent of the silicotic subjects .

That an uneven distribution of the insp air is an outstanding feature of emphys is borne out clearly by the present st It may be found, however, in other dise also, notably in some cases of bronc asthma (cp. Bates, 1952; Colldahl & Lun 1952). In patients with small lung volu *e. g.* cases of more pronounced silicosis, t is usually only slight evidence of uneven distribution. Although some correlatio found between the air content of the l (*i. .e* functional residual capacity) and disturbances in air distribution, the l cannot be regarded as caused simply b increased lung volume, as wide differe in nitrogen washout volume are found tween patients with approximately the s functional residual capacity (Fig. 4). supporting this contention is the evid of increasingly uneven air distribution increasing age, in spite of the remark constancy of the functional residual capa throughout the years. This was found Bates & Christie, 1950), Briscoe, Beck & Rose (1951), Briscoe (1952), Fo Cornish & Kety (1952), Greifenstein, K Latch & Comroe (1951—52) as well a the present study.

The disturbances in intrapulmonary distribution may be expressed quantitati as a poorly ventilated space of a ce magnitude. This calculation, which s very useful in studies of equilibrium closed system (Briscoe, 1952) has not found reliable with the open-circuit p dure used in the present study. The culties are partly due to the deflection c curve caused by nitrogen elimination the body. This difficulty has been cir

ted by Hickam, Blair & Frayser (1954), ir & Hickam (1955), who use helium as .cator gas and follow the elimination after liminary respiration in a 50 per cent um-oxygen mixture. Their method, ch has the drawback of being rather ly, seems specially well-adapted to the ermination of the volume of the most rly ventilated part of the lungs.

SUMMARY

The nitrogen elimination from the lungs ing oxygen breathing was followed in 50 mal subjects and in 150 patients with monary disease.

An alveolar nitrogen concentration of 2.5 cent was reached by the normal subjects 5.5 minutes or less. The total ventilation d from the start of breathing oxygen il this effect was obtained, the nitrogen shout volume, averaged 24 litres, with litres as the upper normal limit.

The nitrogen washout volume was found increase with increasing age.

n 50 emphysematous subjects the mean ue for the nitrogen washout volume was litres; in 50 patients with chronic bron- is without emphysema, 32.5 litres; and 50 silicotic subjects, 30.9 litres.

A rather crude correlation was found be- en nitrogen washout volume and func- al residual capacity.

t is suggested that the pulmonary emp- ig rate of Cournand *et al.* may be more sitive for discriminating normal from hological air distribution if the alveolar rogen concentration is measured after a tain ventilation (*e. g.* 45 litres) rather n after a certain time (*e. g.* 7 minutes) originally proposed.

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DETERMINATION OF SODIUM IN DRIED UMBILICAL CORD TISSUE BY FLAME PHOTOMETRY

By H. H. LAUSEN

From the Department of Pharmacology, University of Copenhagen, Copenhagen, Denmark

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A series of investigations into the conditions of swelling of umbilical cord tissue included determination of the sodium contained in the tissue concerned. Flame photometry was chosen as the most convenient method.

PRINCIPLE OF PROCEDURE

The tissue material, available in dried condition, is soaked in concentrated nitric acid and hydrogen peroxide. After standing at room temperature until the tissue has dissolved, the mixture is diluted with a suitable volume of water, to which has been added *n*-propanol. The sodium concentration of the resulting tissue solution is determined by flame photometry, using sodium chloride standard solutions prepared in a similar way.

Tissue solutions.

The tissue specimens concerned, which have been dried after the swelling, now weighing 10 to 30 mg, are placed in a 10 ml graduated flask (see, however, below), into which is poured 1.0 ml of concentrated nitric acid as well as 0.2 ml of 30 per cent hydrogen peroxide. The flasks are left at room temperature till the next day, when the tissue specimens will have dissolved, forming a turbid fluid, in some instances with a sediment. The solution is diluted somewhat with distilled water. Thereafter 1.2 ml of *n*-propanol is added, and finally distilled water to the mark. The fluid thus prepared often contains a flocculent precipitate, settling rapidly after shaking, thus allowing the fluid to be decanted clear. A slight turbidity seems to no disturbing influence on the flame photom however, *e. g.* by clogging the atomizer. A c of 12 per cent propanol has been found to a suitable and even spray.

The dried tissue specimens weigh, as s 10—30 mg, corresponding to 100—600 mg hy tissue with sodium concentrations ranging 0 to 300 meq/kg. To narrow down proporti the range of concentrations in the tissue solu 5 ml graduated flasks have been used for specimens with a low sodium content, and flasks for tissue specimens with a high s content. The sodium concentrations of the solutions have thereby been kept under 8 Where 5 or 15 ml graduated flasks are use above volumes of nitric acid, hydrogen per and propanol are multiplied by 0.5 and 1.5 r tively, so that these reagents always are u the same concentrations.

The standard solutions

are all prepared from one and the same sodium chloride solution. The volumes requir measured in 10 ml graduated flasks by mea Carlsberg constriction pipettes. Then nitric and hydrogen peroxide and later propanc added in the same quantities as to the tissue tions, and likewise at two stages. Finally, a solution is prepared without sodium chlorid otherwise in the same way. It has as a rul found sufficient to have 3 or 4 concentrati standard solutions, which are always prepare measured in duplicate.

Flame photometry

eckman Quartz Spectrophotometer, model DU Flame Attachment 10300 is used (the old n with chimney and heated spray chamber). lame is a gas flame from bottled gas (Kosan-R) : about 50 per cent butane and about 50 per propane). The pressure is such that the cones —3 mm high, with an oxygen pressure of 20 s of water. The solutions to be measured are ed by means of compressed air with a pressure lbs/sq.in. The emission at 589 mμ is measured, arbitrarily expressed by the units on the ent-transmission scale. The apparatus is ad-l as indicated in Beckman's Bulletin 193-B, lit of the monochromator being adjusted so solution containing 5 meq/l gives an emission —70 units.

Measurements

ter the flame photometer has been adjusted scribed above, the emissions from a series of ard solutions are measured. The measure-s of the series are repeated, until the emission s are stable within 1—2 units. Next a series t more than 10 tissue solutions is measured, then again the standard series. The whole dure is repeated until the emissions of the solutions have been closely fixed in relation se of the standard solutions. Impurities in the zer are easily recognizable during these rements.

e commonly employed technique for deter-g the sodium concentration in plasma dilu-*i. e.* that of alternately measuring plasma dilu-nd standard solution, is not suitable for the nt study owing to the wide range of concen-ns of the tissue solutions.

RESULTS

otting of the emission values obtained he standard solutions against the corre-ding sodium concentrations gave a cur-line (Fig. 1). In sodium determination lasma, the range of concentrations for tandard solutions is so narrow that one

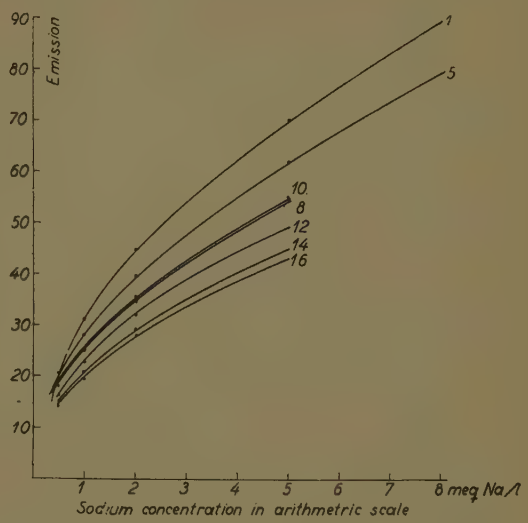

Fig. 1. Standard curves for determination of sodium with a Beckman Flame Photometer.
Abscissa: Sodium concentrations in meq/l, plotted in arithmetic scale.
Ordinate: Emission values in units on the per cent-transmission scale, plotted in arithmetic scale.
The figure shows only 7 of 16 standard curves obtained within 2 hours, and the fan-shaped pattern is due to "drifting" in the apparatus.

must be justified in connecting the points by straight lines.

As it appeared that the curved lines always could be drawn with the same curve ruler, since only their slopes varied, and that "drifting" in the apparatus manifested itself by altered slopes of the curved lines, it was attempted to change these into straight lines. The emission values were in all cases on an arithmetic scale. Plotting of the sodium concentrations on a logarithmic scale gave oppositely curved lines. By plotting the square roots of the concentrations, straight lines were found, whereas plotting of the

cube roots of the concentrations likewise gave oppositely curved lines. It therefore was chosen to calculate the sodium concentrations in the tissue solutions on the basis of the rectilinear standard curves resulting when the emission values from the standard solutions were plotted against the square roots of the sodium concentrations (Fig. 2). The curves were drawn on ordinary graph paper. The emission (10 units = 2 cm) was plotted as ordinate, and the square root of the sodium concentration as abscissa. Concentrations of 0.5, 1.0, 2.0, and 5.0 meq Na/l, for instance, were set out at 3.54, 5.00, 7.07, and 11.2 cm respectively, as it was necessary to multiply the square roots by 5 to obtain suitable slopes of the lines. The general expression used for calculating the abscissa values is thus $5 \times \sqrt{[Na^+]}$ cm. The sodium concentration of a tissue solution was thereafter calculated by reading on the abscissa axis the number of centimetres that corresponded to the emission measured. The concentration was now found by dividing the centimetre distance by 5 and squaring the quotient (the general expression in the calculation is $(0.2 \times \text{cm})^2$). The calculations required are easily performed by a single adjustment of an ordinary 25 cm slide-rule.

Changes of the standard curves from day to day, as well as drifting in the apparatus will also for the rectilinear curves manifest themselves by altered slopes.

The sodium concentration of the tissue specimen before drying is calculated by multiplying the concentration of the tissue solution by its volume (*i. e.* 5, 10, or 15 ml). This quantity is divided by the weight of the tissue specimen (before drying), expressed in grams, which gives μeq Na/g tissue.

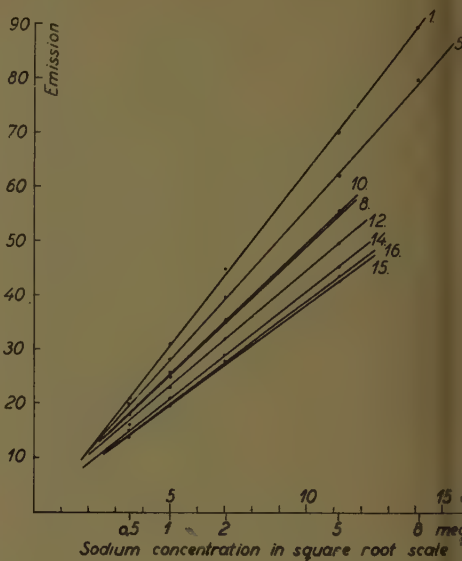

Fig. 2. The curved lines of Fig. 1 convert straight lines by plotting emission values (or as in Fig. 1) against square roots of soc concentrations.

DISCUSSION

All the standard solutions were pre with the same sodium chloride solutio the swelling experiments the tissue mens examined had been imbibed in volumes of distilled water or of pure so chloride solutions, thus interference in the first instance, potassium, calcium phosphate should be avoided. Various ces of error, such as differences in visc surface tension, hydrogen ion concentr salt concentration, adsorption to glass *etc.* should likewise be eliminated by th form compositions of tissue solution standard solutions.

With regard to converting the curvi standard curves into straight lines, the ticability has merely been ascertained used as an analytical aid, which was

rly useful for determining low sodium centrations. The problem has not been her investigated.

SUMMARY

A method is suggested for flame-photoric determination of sodium in dried bilical cord tissue, the tissue being disved in concentrated nitric acid and hydro- peroxide by leaving at room temperature the next day.

Flame photometry was carried out with Beckman Quartz Spectrophotometer with Flame Attachment 10300. The curved lines resulting by plotting parallel values for sodium concentration and emission proved to be convertible into straight lines by plotting the emission values against the square roots of the corresponding sodium concentrations.

ACKNOWLEDGMENT

I wish to thank Professor Eggert Møller, M. D., for permission to use the flame photometer in the Medical Out-Patient Department of the Rigshospital.

STUDIES ON SERUM PROTEINS IN PREGNANCY

By W. von STUDNITZ

From the Department of Clinical Chemistry and Obstetrics and Gynaecology, General Hospital Malmö, Malmö, Sweden

(Received for publications September 7, 1955)

In recent years the changes in serum protein distribution during pregnancy, *i. e.* the decrease in the albumin fraction and in the ratio of albumin to globulin, have been studied in greater detail by means of quantitative electrophoresis.

Lagercrantz (1945), Longsworth, Curtis & Pembroke (1945) used the Tiselius apparatus; Levens (1952), Pfau (1954), and others the microelectrophoresis apparatus of Antweiler; Pfau (1954) and Wolf (1954) paper electrophoresis.

The purpose of the present study was to form a clearer opinion of the behavior of the serum proteins in healthy women in various months of pregnancy, in order to secure normal or reference data for the investigation of pathological changes in serum during pregnancy, and, secondly, to demonstrate changes in the α_1 globulins and hitherto unknown changes in the β_1 and β_2 globulins in various stages of pregnancy.

MATERIAL

The material consisted of sera collected in various months of pregnancy from women without any demonstrable signs of disease. The material for the investigation of the behavior of the β_1 and β_2 globulins consisted of 20 sera of healthy pregnant women in the tenth month.

Table I. *Number of women studie the various months of pregnancy.*

Month	No. of cases
II	6
III	10
IV	14
V	12
VI	14
VII	12
VIII	13
IX	10
X	10
X	(20)
	101 (121)

The distribution of the samples amon various months of pregnancy is given in Ta The control material consisted of sera fro registered donors (male and female). The c sera were not divided according to sex, no ence hitherto being known to exist betwee protein spectra for women and men.

METHODS

1. Paper electrophoresis ,as modified by L Laurell & Skoog (1955).

Buffers used:

A. (pH 8.6) 1.38 g diethylbarbiturate, sodium diethylbarbiturate are dissolved in d water to a total of 1 liter.

B. (pH 8.6) 1.38 g diethylbarbiturate, sodium diethylbarbiturate and 0.384 g calciu

Table II. *Distribution of serum total proteins, albumins, and globulin fractions in the various months of pregnancy. All values are given as g per 100 ml of serum.*

	Total protein	Album.	α_1	α_2	β	γ
Normal mean	7.1	4.53	0.34	0.50	0.85	0.87
S. D.	± 0.26	± 0.18	± 0.036	± 0.051	± 0.083	± 0.11
Month						
II	7.1	4.36	0.38	0.56	0.74	0.94
III	7.0	4.43	0.40	0.57	0.73	0.96
IV	6.8	4.18	0.40	0.59	0.75	0.92
V	6.6	3.95	0.42	0.58	0.77	0.87
VI	6.6	3.79	0.43	0.62	0.81	0.78
VII	6.5	3.74	0.45	0.61	0.88	0.84
VIII	6.6	3.69	0.45	0.66	1.00	0.86
IX	6.6	3.52	0.46	0.70	0.99	0.83
X	6.8	3.69	0.47	0.67	0.99	0.89
S. D. (X)	± 0.3	± 0.18	± 0.066	± 0.042	± 0.15	± 0.09

are dissolved in distilled water to a total of
er.
Used to separate the β-globulin complex in β_1
β_2 fraction).
Total protein determination according to van
e (corrected according to Kjeldahl).

RESULTS

n order to obtain a clearer idea of the
nges occurring during pregnancy, the
ein fractions were expressed in g per
ml serum instead of as percentages of
total proteins.

ble III. *Distribution of β_1 and β_2 globulin fractions in the tenth month of pregnancy. All values are given as g per 100 ml of serum.*

	β_1	β_2
Normal mean	0.50	0.35
S. D.	± 0.06	± 0.05
Month		
X	0.68	0.32
S. D	± 0.07	± 0.07

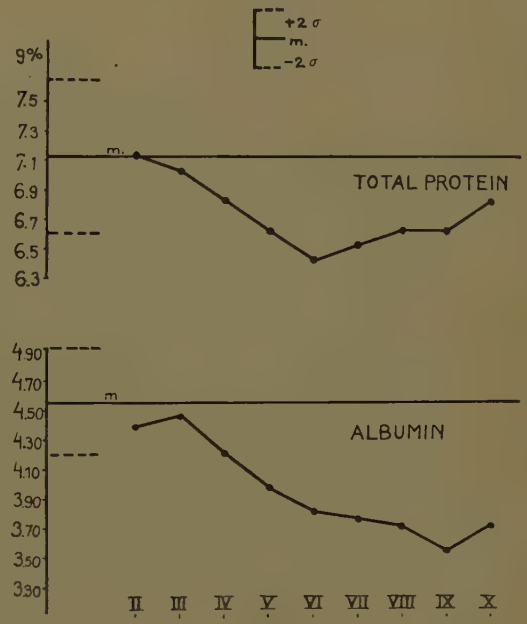

Fig. 1. Concentration of serum protein and albumin during the various months of pregnancy. All values are given as g per 100 ml of serum. The horizontal lines represent the mean values of healthy subjects. The dotted lines give mean values of standard devations.

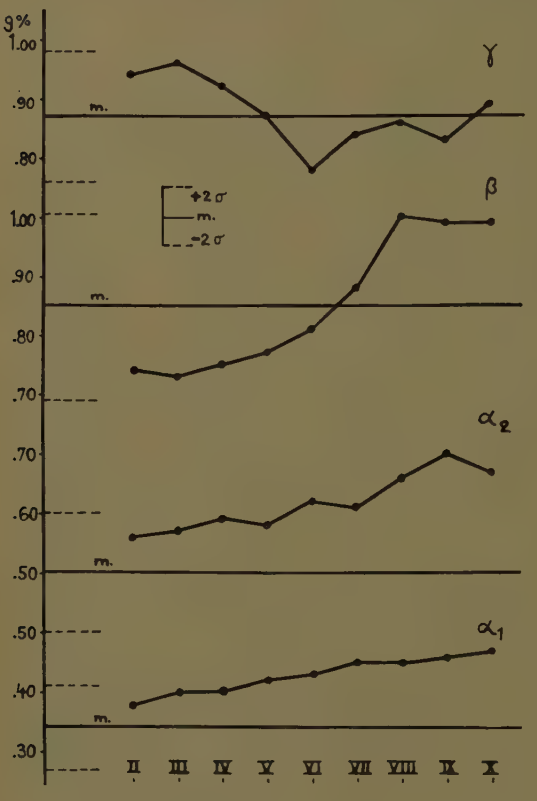

Fig. 2. Concentration of serum α_1, α_2, β, and γ globulin fractions. All values are given as g per 100 ml of serum. The horizontal lines show the mean values of healthy subjects. The dotted lines give mean values of standard devations.

The total proteins showed a tendency to decrease from 7.1 to 6.8 g per cent. The decrease was most pronounced in the sixth month of pregnancy, when the level was 6.4 g per cent.

The fall in the albumin continued in the ninth month of pregnancy when a value of 3.52 g per cent was noted, as against a normal level of 4.53 g per cent.

Ninth month of pregnancy. — The α_1, the α_2, and the β (complex) globulin fractions increased until the end of pregnancy. Du pregnancy the α_1 fraction increased f 0.34 to 0.47 g per cent; the α_2 fraction, f 0.50 to 0.67 g per cent; and the β frac to 0.99 g per cent.

A separation of the β globulin com showed an increase of β_1 globulins from to 0.68 g per cent, while the β_2 globu were constant (Table III). All of these ferences were statistically significant.

The concentration of the gamma glob fraction showed no marked fluctuations.

The results are summarized in Table and III and compared graphically in F 1 and 2.

DISCUSSION

Total proteins. — Wolf (1954) found changes in the total protein value du pregnancy, while Pfau (1954) and Le (1952) reported a decrease, an observa supported by the present investigation.

Albumin. — As in the present invest tion, a decrease was found in the albu by all of the authors referred to in foregoing.

α globulins. — With the exceptio Wolf (1954), all workers in this field studied the α globulin only as a com Masek & Masowa (1950) were the ones to find the complex constant, whil the others found an increase. Wolf f an increase in the α_2 fraction and stre the stability of the α_1 fraction. The pr investigation, however, showed not onl increase in the a_2 fraction, but also a s ficant increase in the α_1 fraction.

As will be apparent, the increase f in the α_2 fraction cannot be expla entirely by the high copper content, w

vs the increase in the protein complex uloplasmin during pregnancy. Normally serum contains about 0.031 g per 100 ml rresponding to 100 micrograms of cop- , which increase to about 0.062 g per ml (corresponding to 200 micrograms opper) during pregnancy. This increase however, not sufficient to explain the ease in the α_2 fraction from 0.50 to 0.67 g 100 ml. It thus appears that other com- ents must contribute to this phenomenon.

globulins. — In the present investiga- , as in earlier studies by other workers, ncrease was found in the β globulin com- . However, all workers in this field have lied the β globulins as a complex only. results of the present investigation wed an increase in the β_1 globulins, but in the β_2 globulins.

globulins. — Masek & Maskowa (1950) Neuweiler (1948) reported an increase he γ globulins during pregnancy. This ervation has, however, not been confirmed others or by the findings in the present stigation.

he pronounced increase in the plasma me during pregnancy is well-known. ough the figures on record vary from author to another, they are all roughly he same order. Thus, Schröder (1949) ribed an increase of 30—35 per cent and n (1949) found the plasma volume to 9 per cent higher during pregnancy than days *post partum.*

is thus apparent that all the fractions thereby the total proteins show an lute increase during pregnancy. The uently stressed significance of this abso- increase should, however, not be over- nated, for it is not the absolute amount but the concentration of a substance in the blood that is of physiological importance. The changes in the serum proteins during pregnancy consist of a decrease in the concentration of the albumin and of the total proteins and in an increase in the concentration of α_1, α_2 and the β_1 globulins.

The increase found in the albumin and the total proteins during the tenth month tallies well with Caton's finding of a decrease of the plasma volume in that month.

The increase in the alpha and β globulin fractions will be discussed in detail in future papers on the changes in the lipoproteins and the glycoproteins during pregnancy.

SUMMARY

Observations made in an investigation of serum proteins in pregnancy suggested the following conclusions.

1. The concentrations of the total serum proteins and serum albumin decrease significantly during pregnancy.

2. The α_1, α_2, and β_1 globulins increase significantly.

3. No significant changes occur in the γ and β_2 globulins.

ACKNOWLEDGMENT

My thanks are due to Miss Nancy Skoog for skilful technical assistance.

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STUDIES ON SERUM LIPIDS AND LIPOPROTEINS IN PREGNANCY

By W. von STUDNITZ

From the Departments of Clinical Chemistry and Obstetrics and Gynaecology, General Hospital of Malmö, Malmö, Sweden

(Received for publication September 7, 1955)

t has long been known that the serum increases during pregnancy. The first orts of this change were made by Becerl (1845) and Virchow (1847). Since n this phenomenon has been the subject many investigations, which have shown t the phospholipids, the cholesterol, the tral fat and the fatty acids likewise inase during pregnancy. Recent research also revealed that in fasting individuals of the serum fat is bound to protein. ese investigations were carried out with ving boundary electrophoresis (Blix, elius & Svensson, 1941).

The following methods have proved useful the investigation of the lipoproteins of um:

) Chemical fractionation according to Cohn group (1950), 2) ultracentrifuga-, the Gofman group (1949), 3) paper trophoresis, Swahn and others (1953), preparative zone electrophoresis with ying techniques (Carlsson, 1954; Kunkel Slater, 1952).

As to the behavior of the lipoproteins ing pregnancy, Russ, Eder & Barr (1954), used Cohn's ethanol fractionation, died the various protein fractions for their tent of cholesterol and phospholipids in from maternal blood samples collected during delivery, and Gross & Weicker (1954) described paper electrophoresis diagrams of lipids from samples from two pregnant women.

It appears that, apart from these two investigations, little if any, attention has been directed to changes in the lipoproteins in pregnant women.

The purpose of the present investigation was to chart the course of the changes in lipoproteins throughout pregnancy in healthy women, in order to secure a normal curve that might be useful in the evaluation of lipoprotein patterns of pregnancy in association with certain diseases. For purpose of completion the serum phospholipids and cholesterol were also determined.

It should be mentioned that, of the methods listed above, Gofman's ultracentrifugation and preparative zone electrophoresis are less suitable for routine analyses. Gofman's method requires expensive laboratory equipment and is technically difficult. Preparative zone electrophoresis is time-consuming. Cohn's chemical fractionation with precipitation of the proteins and subsequent determination of the fat in the individual fractions is likewise fairly tedious. In addition, the value of this method is restricted by the fact that the limits of the precipitation zones

depend on the protein composition of the original material. In other words, in the investigation of pathological sera there occurs a certain degree of overlapping between the various fractions.

It was decided to use Swahn's (1953) paper electrophoresis technique in the present study since it is fairly simple and gives reproducible lipoprotein values for both normal and pathological sera. A disadvantage of the method is that the high molecular lipoproteins are partially adsorbed to filter paper. In order to minimize this sorption, a short migration distance w chosen. In spite of this a good picture the distribution of the lipoproteins in two main fractions, *i. e.* the α and β fr tions, is obtained. The method yields information on the quantitative distribut of the fat in the α fraction, which, in t method, comprimises albumin, α_1, and However, the major portion of the fat the α fraction belongs to the α_1 lipoprote

The changes of the serum protein patt during pregnancy were reported in previous paper (v. Studnitz, 1955).

Table I. *Number of women studied in the various months of pregnancy.*

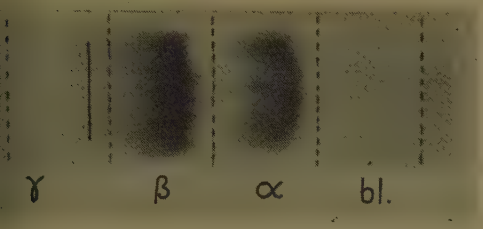
Month	Cases
II	6
III	10
IV	14
V	12
VI	14
VII	12
VIII	13
IX	10
X	10
	101

MATERIAL

The material consisted of sera from 101 hea pregnant women (Table I) and 10 control from healthy non-pregnant women aged 18 35 and selected at random during the autum 1954 and the spring of 1955. The normal va for cholesterol, phospholipids and their stan deviation were calculated on the basis of from large series investigated in our laborat

Table II. *Distribution of serum total lipids, lipoproteins, phospholipids, and ch esterol in the various months of pregnancy. All values are given as g per 100 ml of serum.*

	Total lipids	α	β	γ	Phospholipids	Choles
Normal mean	664	224	374	63	242	20
S. D.	± 124	± 54	± 93	± 34	± 28	± 3
Month						
II	725	264	380	78	245	17
III	653	245	347	63	258	19
IV	729	239	413	71	289	21
V	899	329	451	113	324	21
VI	813	260	476	71	335	24
VII	972	294	589	83	364	24
VIII	934	257	556	126	355	26
IX	944	303	610	60	351	26
X	966	326	597	45	398	2
S. D. (X)	± 145	± 42	± 98	± 41	± 111	±



1. Lipid patterns. Separation distance 6 cm.

METHOD

. Paper electrophoresis according to Swahn 53) as modified by Laurell, Laurell & Skoog. ntity of serum used: 0.02 ml. Separation ance of 6 cm, which corresponds to a separation of 3 hours: see Fig. 1.

. Determination of total fat *ad modum* Swahn 53). The method was slightly modified. Before ing the serum on the filter paper the latter was hly moistened in order to secure complete tion of the coloring agent. Triolein (Merck) olved in carbon tetrachloride was used as dard. The colored paper strips were washed for 20 minutes and then for 10 minutes in t 100 ml of 50 per cent alcohol.

. Determination of the phospholipids according Petersen (1952).

. Determination of the cholesterol according Abell, Levy, Brodie & Kendall (1952).

ble III. *Distribution of serum lipoprotein fractions in the various months of gnancy. The values are given as per cent of the total lipids.*

	α	β	γ
mal mean	34.2	56.4	9.3
ith			
II	36.8	52.4	10.6
II	39.2	53.2	9.6
V	32.6	56.6	9.8
V	37.1	50.2	12.6
VI	32.7	58.6	8.7
II	34.6	60.6	8.5
II	27.4	59.5	13.5
X	32.0	64.6	6.4
X	33.4	61.8	4.7

RESULTS

The results of the analyses are summarized in Tables II and III.

Total lipids. — During pregnancy the mean values for the total lipids increased from 664 to 996 mg per 100 ml. The difference was statistically significant. This increase ran a fluctuating course with marked dips in the third and sixth months (Fig. 2).

α lipoproteins. — The α fraction also showed an increase from 224 to 326 mg per ml. This increase was likewise statistically significant. This curve parallels that for the total fat with the exception of the fourth month, in which the total fat dropped to a minimum. The minimum in the sixth month was found to be statistically probable. The curve for the relative α values, on the other hand, showed a tendency to decline from 37 to 33 per cent of the total fat.

β lipoproteins. — This curve was of corresponding course, but showed no minimum

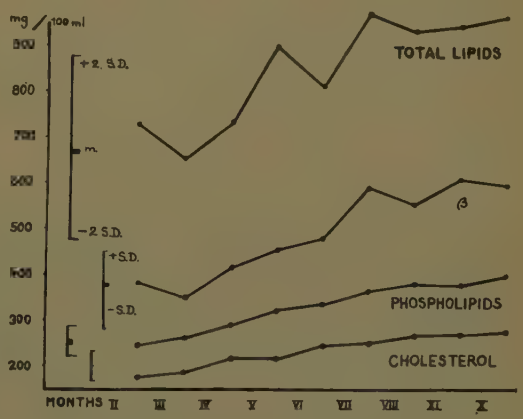


Fig. 2. Curves showing the concentration of total lipids, β lipoproteins, phospholipids, and cholesterol during the various months of pregnancy. Mean values and standard deviations are given on the ordinate.

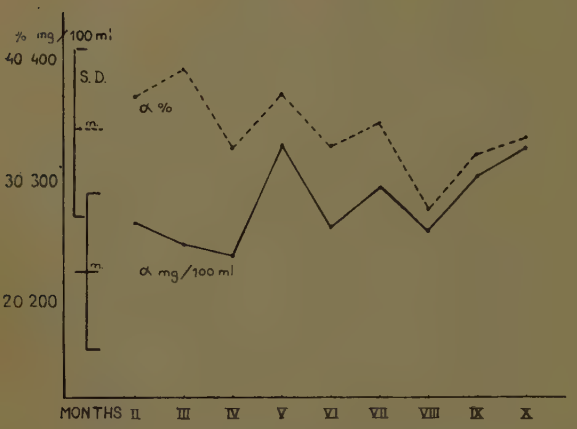


Fig. 3. Curves showing total amounts of α lipoproteins and their per cent of total lipids in the various months of pregnancy. Mean values and standard deviations are given on the ordinate.

in the fourth month. An increase was thus noted in the β lipoproteins from 375 to 597 mg per 100 ml. This difference was significant. The relative values showed an increase from 52 to 62 per cent of the total fat (Fig. 2).

On comparison of the stained electrophoretic strips it was found that the relative rate of migration of the β fraction increased with advancing pregnancy. This is readily seen from the Fig. 4, in which the lipid patterns for various months of pregnancy compared with patterns for non-pregn healthy women. Fig. 5 shows the ra migration of the β fraction of a sample lected shortly before delivery (A and B) the gradual decrease after delivery (C D). (The behavior of the α fraction in case — the mother was not healthy — be described in a later paper on the li and lipoproteins in pregnancy in associa with disease).

The γ fraction. — This fraction cont chylomicrons and adsorbed β-lipoprot It decreased from 10 to 5 per cent tow the end of pregnancy.

Serum cholesterol. — The cholesterol tent gradually increased from 176 to 276 per cent 100 ml. The increase was statisti significant (Fig. 2).

Phospholipids. — The phospholipids creased from 245 to 398 per 100 ml. The ference was statistically significant (Fig

DISCUSSION

It appears that as yet only few atte have been made to find an explanation the increase of serum lipids during nancy. Seitz (1951), for example, sugg

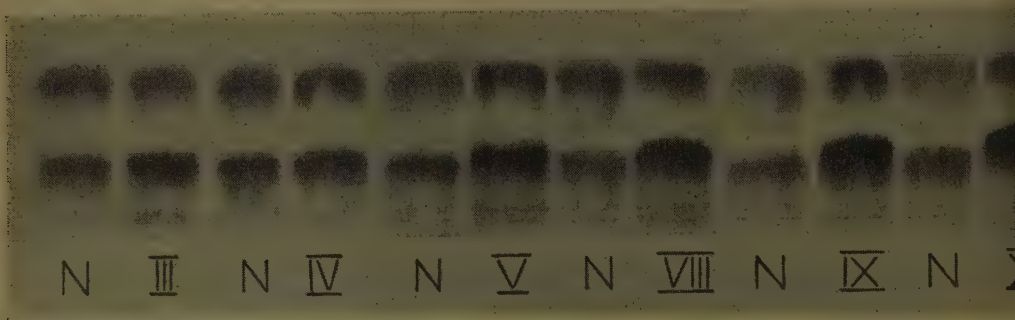
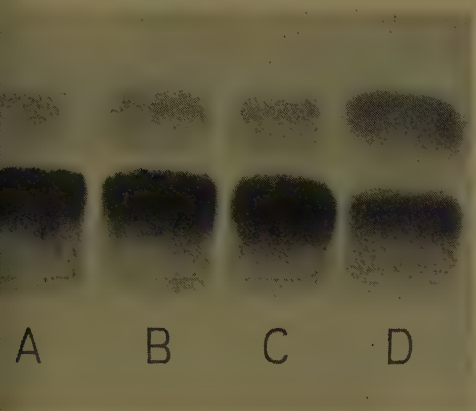


Fig. 4. Electrophoretic diagrams for various months of pregnancy compared with alternating grams for non-pregnant women (N). The increased migration of the β fraction from the fifth m on is readily recognized.



5. Four diagrams of a patient with toxemic pregnancy.
4 weeks *ante partum*
(total lipids 1207 mg per 100 ml serum).
2 days *ante partum*
(total lipids 1450 mg per 100 ml serum).
2 days *post partum*
(total lipids 1005 mg per 100 ml serum).
5 weeks *post partum*
(total lipids 630 mg per 100 ml serum).
e the graduate normalization of the rate of the β fraction.
(As to the α fraction see text.)

t the increase might be due to the fat uirement of the fetus, but he offered no lanation for the underlying mechanism. objection that can be raised against this ory, however, is that lipemia is still more nounced in toxic pregnancy.

In recent years increasing attention has n directed to the influence of estrogens, pophyseal and suprarenal hormones on fat metabolism.

Estrogens. — Barr, Russ, & Eder (1952) l Russ, Eder & Barr (1954) have shown t the absolute and relative concentration the α lipoproteins in women aged 18 to years was higher than that in men of responding age. This difference was ascribed to the effect of estrogen. The administration of estrogenic substances to patients with atherosclerosis was followed by an increase of the α- and a decrease of the β-lipoproteins. The gradual increase of the α lipoproteins during pregnancy may be correlated with the increase in production of estrogen.

Suprarenal hormones. — Di Luzio, Shore, & Zilversmit (1954) found that the administration of cortisone increased the cholesterol and the phospholipid levels in experimental animals. In humans Adlersberg (1951) observed not only these changes but also a decrease in neutral fat. This increase in the serum lipids during pregnancy can thus possibly be assessed with the increased production of corticoids (Venning, 1946; Bayliss, 1955).

In an investigation of the excretion of corticoids by nine women, Venning (1946) obtained a curve with two maxima in the second and in the third months as well as in the seventh and ninth months. The course of this curve thus resembled the shape of that found for the total lipids in the present investigation (Fig. 2).

The increase in the excretion of gonadotropin in the second and third months is known from the work of Venning (1946) and others. Whether the increase in gonadotropin is responsible for the decrease in the total fat in the third month — a decrease also reported earlier by Peters (1951) — is debatable.

The hormonal interplay during pregnancy is very intricate. The influence of progesterone must also be taken into account as well as its apparant interaction (Kayser, 1955) with the estrogens.

Observations made in the present investigation of the lipid changes during pregnancy may suggest that the increase in the β-lipoproteins might be related to an increased production of corticoids. The increase in the α-lipoproteins may be ascribed to the increased production of estrogens.

In the present investigation the observation was made that the migration rate of the β-lipoproteins in advanced pregnancy was higher than that found in a non-pregnant condition. This increase in rate of migration of the β lipoproteins may be due to a change in their composition but has not yet been definitely cleared up. After addition of free fatty acids to serum the migration rate of the β-lipoproteins and α-lipoproteins increase (Laurell, 1955) during pregnancy. However, the migration of the α-lipoproteins remains unchanged. An observation (Ray, 1954) that may be of interest in this connection is that the migration rate of the β-lipoproteins increases upon storage. This has been explained as a result of the oxidation of the phospholipids.

Gross & Weicker (1954) described an increase in the γ lipoprotein fraction during the later part of pregnancy and stressed the similarity between the pattern of these sera and postprandial hyperlipemia. No increase was found in the present series. The data obtained here confirmed the crease — described by Boyd (1934) Peters (1951 — in the phospholipids cholesterol during pregnancy.

The increase in the α_1 and α_2 globuli pregnancy described elsewhere (v. Stud 1955) can be ascribed partially to the inc of the corresponding lipoprotein frac The changes of the glucoproteins d pregnancy will be the subject of a sep paper to be published later.

SUMMARY

101 sera of healthy pregnant wome various months of pregnancy were inv gated for changes in fat and lipoprotei

A significant increase was found in total lipids, the α and β lipoproteins, cholesterol, and in the phospholipids d pregnancy.

Furthermore, the migration rate of t lipoproteins was found to increase with vancing pregnancy.

It is tentatively suggested that the cha in the fat fractions might be due, at partly, to hormonal changes in pregnan

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PROTEIN-BOUND IODINE (PBI) — INVESTIGATION INTO BARKERS DISTILLATION METHOD

By T. FRIIS

From the Central Laboratory, Frederiksberg Hospital, Copenhagen, Denmark

(Received for publication Sept. 9, 1955)

Several investigations have proved the concentration of protein-bound iodine in serum to be an asset in diagnosing thyrotoxicosis and myxedema (Barker, Humphrey & Soley, 1951; Rapport & Curtis, 1950 and others). In the writer's material (1954), the concentration in persons with normal thyroid condition has been found to lie between 4 and 8 μg/100 ml, with a mean value of 6.12 μg/100 ml and a standard deviation of $\pm$ 1.3 μg/100 ml.

The present study deals with some investigations into the modification of Barker's method of distillation used in this laboratory.

METHOD

The *principle* in iodine analysis is as follows: After precipitation and washing, the serum proteins are digested by concentrated sulphuric acid and chromic trioxide. The iodic acid thus formed is reduced by phosphorous acid to hydric iodide and distilled into alkaline fluid, where it becomes bound as iodide. In measuring the amount of iodide, advantage is taken of the fact that iodide has a catalytic effect on the reduction of ceric ammonium sulphate by arsenic trioxide. The amount of ceric ammonium sulphate reduced in a given period is dependent on iodide concentration. The reduction rate is read by photocolorimeter, the ceric ammonium sulphate being yellow and the reduced product colorless. The iodide concentration is determined by plotting a standard curve on the basis of known iodide solutions.

The investigation covers the following asp (1) the effectivity of the washing procedure the influence of temperature on the effectivi digestion, (3) the effectivity of the distillation the dependence of the reduction of ceric ammo sulphate on temperature and time, and (5 evaluation of the accuracy of the analysis m in our hands.

Special analysis criteria

In order to obtain results of practical cl significance, it is essential that the patients sh not have ingested large amounts of iodine-i during the previous three weeks, or have been jected to X-ray examination with contrast stances containing iodine (the concentration o protein-bound iodine is increased for up weeks after urography, 3 months after cholec graphy, 9 months after bronchography, and 3 years after myelography (Rapport et al., Starr, Petit, Chaney, Rollmann, Aiken, Jamies Kling, 1950). This would cause the values increased considerably so that the results v become useless from the clinical point of vie

Blood was taken with ordinary dry ster venous puncture needles. 10—15 ml venous was taken into a clean test tube, which was c immediately with a clean rubber stopper. It the greatest importance that the person takin blood should not have iodine on his fingers there must be no open iodine flasks nearby. parison of blood taken with both ordinary sterilised needles and with needles which had rinsed in redistilled water showed that there no difference in the analysis results.

e analyses were carried out in a special room e there was no dust, no smoking or no work other types of analysis. These precautions taken to avoid the very considerable danger ntamination with iodine. Tubes containing es, reagents and sera were never allowed to uncovered.

ie tubes and apparatus were washed in chrome- uric acid for 24 hours, after which they were l three times with distilled water and three with water redistilled in a glass still. The r stoppers were blood in soap solution and after rinsed several times with redistilled r.

Reagents

) *¾-normal NaOH* (Merck's analytical pro- . 30 g NaOH is dissolved in 1 litre glass- tilled water (Barker 1948).

) *1.25 per cent zinc sulphate solution.* 12.5 g O_4, $7H_2O$ (Merck's analytical product) is lved in 125 ml ¼-normal H_2SO_4, and diluted litre with redistilled water. The two solutions alanced until 50 ml of zinc sulphate solution res between 6.7 and 6.8 ml of sodium hy- ide solution to show a permanent pink to lphthalein (Barker, 1948).

) *70 per cent sulphuric acid.* 250 ml concen- l sulphuric acid (Barker's 96 per cent ana- l As- and N-free product) is boiled for 20 tes with 2 ml concentrated hydrochloric acid, der to remove any existing iodine and hydric e (Taurog and Charkoff, 1946). Redistilled r is then added to a strength of 70 per cent nl redistilled water to 200 ml acid).

) *60 per cent chromic trioxide.* 300 g chromic ide is dissolved in redistilled water and diluted 500 ml (Barker, 1948)). Chromic trioxide rations, particularly analytical products, often in iodine. It is our experience that it is not ble to remove the iodine from chromic trioxide he boiling procedures mentioned by Moran 2), since the temperature does not become iently high. Technically coarse products are iodine-free (*e. g.* Bie & Berntsen's and rs' technical products).

) *50 per cent phosphorous acid.* 250 g phos- ous acid (Hopkins & Williams' analytical pro- duct) is dissolved in approximately 200 ml re- distilled water and 2 ml 33 per cent peroxide of hydrogen is added (Moran, 1952). The solution is heated in 2 hours. When the temperature exceeds 180° C water is added or else the phosphorous acid is oxydized to phosphoric acid. Finally, the solu- tion is diluted to 500 ml with redistilled water.

(6) *Concentrated "trap solution".* 3.75 g As_2O_3 (Mercks's analytical product) is dissolved in 100 ml 5-normal NaOH (Merck's analytical product) and diluted with redistilled water to 500 ml (Moran 1952).

(7) *Diluted "trap solution".* 80 ml concentrated "trap solution" + 920 ml redistilled water.

(8) *Arsenious acid solution.* 3.0 g As_2O_3 Merck's analytical product) and 87.5 g NaCl (analytical product of British Drug Houses) are dissolved in 200 ml ¼-normal NaOH. 1 litre redistilled water and 764 ml 96 per cent boiled sulphuric acid are mixed. After cooling the arsenious acid mixture is poured into the sulphuric acid. The mixture is diluted with redistilled water to a volume of 2 litres (Querido, Leiden).

(9) *Ceric ammonium sulphate solution.* 12 g ceric ammonium sulphate (Hopkins & William's analytical product) is dissolved in 500 ml 3½-nor- mal sulphuric acid (Barker, 1948).

Procedure

2 ml serum is transfered to a 50 ml centrifuge tube by means of a pipette. The serum proteins are precipitated by Somogyi's precipitating reagents (Somogyi, 1930).

16 ml zinc sulphate and 2 ml sodium hydroxide solution are added, the tube shaken and covered, then allowed to stand for 20 minutes. The precipitate is washed four times with 25 ml redistilled water, being centrifuged between each washing. In this way the content of inorganic iodide is removed from the serum (Barker, 1948).

The precipitate is dissolved in 3 ml 70 per cent sulphuric acid and transferred three times with 3 ml 70 per cent sulphuric acid and 5 ml redistilled water to a special double-necked digestion flask (Fig. 1). It is then allowed to boil after the addi- tion of 3 ml chromic trioxide and 5 ml redistilled water. Boiling is interrupted when sulphuric acid

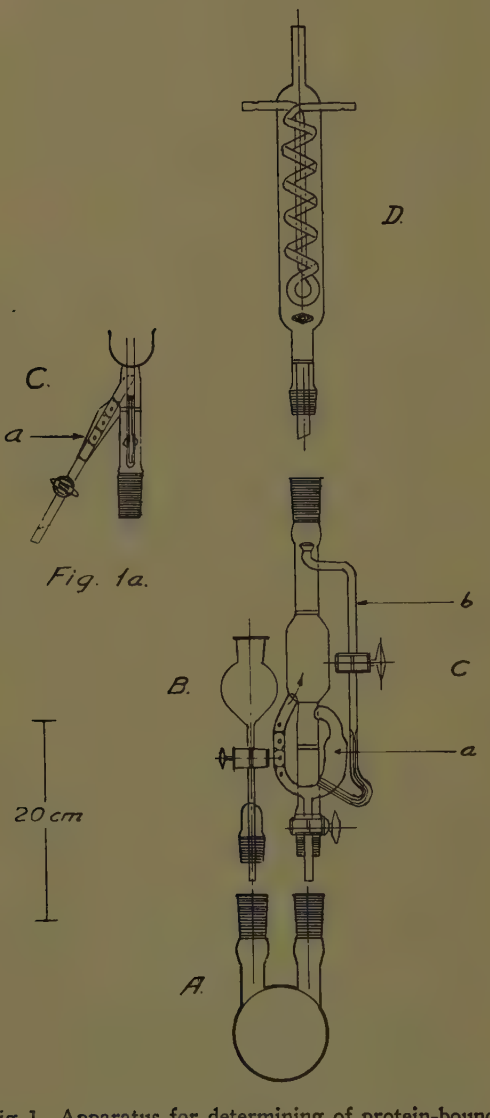


Fig. 1. Apparatus for determining of protein-bound iodine

A: Digestion flask. B: Separating funnel. C: Chaney's still.

a. Trap. b. Return tube. D. Condenser.

Fig. 1 a shows the trap from the side view.

fumes appear, and 15 ml redistilled water ad reduce the boiling point. Digestion is repeate sulphuric acid fumes again appear. The dig procedure takes about 20 minutes in all, by time the protein-bound iodine is released as volatile iodic acid (HIO_3). Digestion is c out in a special apparatus with micro burn that eight analyses can be digested at a tir

Distillation is carried out in Chaney's (Chaney, 1940) (Fig. 1.). 25 ml redistilled is added to the digestion flask containin digested product. The flask is attached to th and 2 ml "trap solution" is transferred by to the trap of the still and 5 ml phosphorou to the separating funnel. A condenser is pla the upper opening of the still. Stopcocks and g glass joints are moistened with redistilled wa

The flask is heated, and when the liquid bo stopcock to the separating funnel is opene phosphorous acid is blown carefully into the stion flask by means of a rubber balloon (pressure in the flask). The liquid becomes and boils rapidly, and the reduced iodic a distilled into the alkaline trap solution and as iodide. The return tube of the still is open distillation carried out for exactly 10 minute completion of distillation, the stopcock on th is opened and the trap solution is drained 50 ml measuring flask. Boiling is disco and the still rinsed three times with distilled The distillate is diluted in the flask to 50 m redistilled water. The PBI of 2 ml serum present as iodide.

As mentioned previously, the amount of is determined by the catalytic effect on the re

$$2\ Ce^{++++} + H_2AsO_3 + H_2O = 2\ Ce^{+++} + I^- + 2\ H^+;$$

Ce^{++++} is yellow and Ce^{+++} col

The determination procedure is as f Twice 5 ml distillate are transferred to t tubes by means of a pipette. 1 ml arsenio solution is added and the tubes placed in bath at 29° C. After 20 minutes' incubation, ceric ammonium sulphate solution is add tubes are shaken, and 28 minutes later th intensity of the mixture is read in a Beck spectrophotometer at 430 mμ. Reading

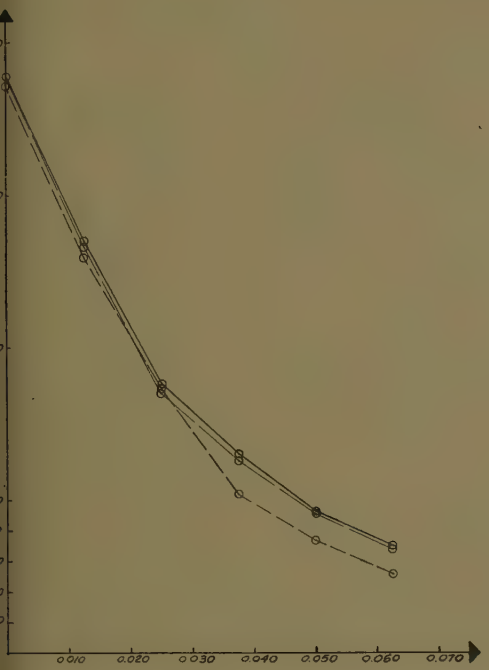


Fig. 2. Three standard curves.
Abscissa: Total amount of iodide in μg.
Ordinate: Extinction.
Temperature: 29° C.
Reaction period: 28 minutes.

ıdard curve gives a measurement of the amount odide in the test tube. This multiplied by 10 ıtent of protein-bound iodine in 2 ml serum) by 50 (content of protein-bound iodine in 100 serum) gives the amount of protein-bound iodine ıg/100 ml, with an accuracy of ¼ μg/100 ml. When many tubes are to be read, ceric ammo- n sulphate is added to tube no. 1 at zero time, ube no. 2 after 1 minute, to tube no. 3 after 2 utes, and so on. After 28 minutes tube no. 1 ead, after 29 minutes tube no. 2, and so on. Thus ubes can be read in one series. In other words, series can include 13 analyses or 6 tests with licate determinations, in addition to blank values. he present work, the blank value varied from 1 μg/100 ml. One series takes eight hours.

Determinations of standard curve: The following aqueous solutions are prepared:

Solution containing	0.025 μg	I^- per ml
»	0.020 μg	»
»	0.015 μg	»
»	0.010 μg	»
»	0.005 μg	»
»	0.000 μg	»

First a solution containing 100 μg I^- per ml is prepared (130.8 mg KI dissolved in 1 litre redistilled water). 1 ml of this solution is diluted to 1 litre containing 0.1 μg I^- per ml. The other dilutions are made from this solution. All dilutions must be freshly prepared.

10 ml of the solutions are transferred by pipette to test tubes and 10 ml diluted "trap solution" added. After mixing twice 5 ml are transfered by pipette from each tube to two test tubes containing 1 ml arsenious acid solution. The twelve tubes are placed in water bath, and finally 0.25 ml ceric sulphate ammonium solution is added. The test tubes now contain ¼ of the amount of iodide in 10 ml of the respective standard solutions. The period of standing and the method of reading are exactly as for the analyses.

It is necessary to carry out new standard determinations when fresh reagents are used in determinations of the amounts of iodide in the distillate. Fig. 2 shows some standard curves.

AUTHOR'S INVESTIGATIONS

1. *Investigation into the washing procedure and the effect of addition of KI and thyroxin on the analysis results*

Connor and co-workers (1949) have stated that it is sufficient to wash precipitate once in order to remove the non-protein bound iodine an iodide. We have gone further into this aspect and have also studied to what extent the addition of KI and thyroxin affects the analyses results.

The procedure was as follows: After the precipitate had been precipitated with

Table I. *Iodide in washing water and PBI in serum immidiately after addition of varying amounts of potassium iodide to 2 ml serum.*

KI added	I^- in 1st washing	I^- in 2nd washing	I^- in 3rd washing	I^- in 4th washing	Increase in PBI in µg/100 ml
0	+	+	0	0	0
0	+	+	+	0	0
0	+	0	0	0	0
0	+	0	0	0	0
0	+	+	0	0	0
0	+	+	0	0	0
0.2 µg I^-	+	+	+	0	0
0.2 µg I^-	+	+	+	0	0
1.0 µg I^-	+	+	+	0	0
1.0 µg I^-	+	+	+	0	0
10 µg I^-	+	+	+	+	23
10 µg I^-	+	+	+	+	32
100 µg I^-	+	+	+	+	27
100 µg I^-	+	+	+	+	31
200 µg I^-	+	+	+	+	6
200 µg I^-	+	+	+	+	9
1000 µg I^-	+	+	+	+	30
1000 µg I^-	+	+	+	+	37

Somogyi's reagents, it was washed with 25 ml redistilled water and centrifuged. This process was repeated four times. 5 ml was taken out by pipette from each washing water and its content of iodide measured. It will be seen from Table I that it was necessary to wash the precipitate four times before the washing water was free of iodide. It will also be seen from the table that when 10 µg I^- or more was added to 2 ml serum (500 µg/100 ml) the analysis results were higher, since the washing procedure had not been able to remove the iodide added. This agrees with the findings of other workers that ingestion of large amounts of iodide increases the results of analysis (Rapport *et al.*, 1950 and others). It will be seen that there was no proportionality between amount of iodide added and the increa PBI.

The addition of thyroxin gave quite a ferent effect. 0.2 µg thyroxin iodine added to 2 ml serum in 10 cases (76.5 m thyroxin (Hoffman la Roche) dissolve 496 ml redistilled water + 4 ml ¾-no NaOH: This solution contained 0.1 iodine per ml. The solution was diluted times). The ten different sera were anal before and immediately after the add of thyroxin. The results will be seen Table II, which shows that 60 to 110 cent of the thyroxin iodine was recov In other words, the washing procedure c not remove, or could only partially rem the thyroxin added, whilst it could re completely up to 10 µg I^-. The reas presumably that thyroxin quickly bec bound to the serum proteins, either w or partially, in contrast to I^-. Brüg

Table II. *Effect of addition of 0.2 thyroxin iodine to 2 ml serum (= 10 100 ml) (10 different sera).*

Serum	PBI in µg/100 ml before addition		PBI in µg/100 ml after addition	Difference in µg/100 ml	per cent of theoretical
1	5.5	5.5	10.5	5	
2	8.5		18.75	10.25	1
3	6.0		15.0	9.0	
4	6.5		14.0	7.5	
5	5.75	5.25	17.0	11.5	1
6	6.5		12.75	6.25	
7	6.25	5.75	12.5	6.5	
8	5.5		14.5	9.0	
9	6.25	6.25	14.25	8.0	
10	4.0	4.25	12.0	8.0	

ble III. *Significance of temperature during digesting process.*

mpera-ture	Formation of sulphuric acid fumes	No. of heatings	PBI in µg/100 ml in the same serum		
130°	None	2	3.5	3.25	
—160°	Commencing	2	5.5	5.0	6.0
—160°	Commencing	1	5.25	4.5	4.75
80°	Strong	2	3.5	2.75	

mber (1943) and Man, Smirnow, Gildea Peters (1942) have carried out similar s and recovered 90 to 100 per cent of the roxin iodine added.

Effect of temperature on the effectivity of digestion

t is stressed in the literature — Barker 48), Moran (1952) — how important s that the precipitate be digested twice, e otherwise the protein-bound iodine will be liberated as iodic acid and the existing ride cannot be expelled. This latter is ential, since chloride has a catalytic effect the ceric ammonium sulphate. On the er hand, digestion must not be carried beyond the stage where sulphuric acid es appear, as otherwise the temperature omes so high that the iodine escapes rker, 1948; Moran, 1952).

This question has been examined with um on which ten analyses were carried In one case, the precipitate was only ted once, which resulted in slightly lower ues. The results ase shown in Table III.

3. *Effectivity of distillation*

Various workers have discussed the distil-on process (Barker, 1948; Chaney, 1940; ran, 1952 and Taurog *et al.*, 1946). Addition of known amounts of iodide to the digested product often gave low "recovery values" (70 to 80 per cent) when pure phosphorous acids were used for reduction and sodium hydroxide was used as "trap solution" (Barker, 1948, Taurog *et al.*, 1946). However, if hydrogen peroxide is added to the phosphorous acid (Moran, 1952), the "recovery percentage" is improved considerably. The reason is stated to be that hydrogen peroxide increase the reducing ability of the phosphorous acids. This, however, does not appear to be obvious. — Where iodic acid is only reduced to iodine, this can be bound by the trap solution as iodide and iodate. However, the latter does not affect the reaction of the ceric ammonium sulphate and thus it cannot be determined. On the other hand, if the iodic acid is reduced to hydric iodide, this can be bound by the trap solution as iodide. Barker (1948) used sodium sulphite as trap solution and pure phosphorous acid for reduction, and thereby obtained a satisfactory recovery percentage. However, in these circumstances, sulphuric

Table IV. *Result of 43 "recovery experiments"*

80— 90% of added iodide recovered	9 cases
90—110% » » » »	24 »
110—120% » » » »	10 »

Table V. *Effect of hydrogen peroxide in phosphorous acid.*

	Serum 1. PBI in µg/100 ml		Serum 2. PBI in µg/100 ml		Serum 3. PBI in µg/100 ml	
$H_3PO_3+H_2O_2$	4.5	4.0	5.5	5.75	6.0	5.75
$H_3PO_3—H_2O_2$	4.5	4.5	5.5	5.0	6.0	6.25

Table VI. *Significance of As_2O_3 in "trap solution".*

	Serum 1. PBI in µg/100 ml		Serum 2. PBI in µg/100 ml	
Trap solution with As_2O_3	7.25	7.0	6.0	5.75
Trap solution whitout As_2O_3	3.5	2.75	2.5	2.5

(In the diluted «trap solution» used for determination of the standard curve, As_2O_3 was not used in the 2. case)

dioxide can be formed, which affects the reduction of the ceric ammonium sulphate.

First the recovery percentage was examined after the addition of 0.1 µg iodide to the digested product. Hydrogen peroxide was added to the phosphorous acid and arsenious acid to the trap solution (see list of reagents). The result is shown in Table IV. Where the recovery percentage is 100, the PBI will be increased by 5 µg/100 ml. Since the analysis result can be read with an accuracy of ¼ µg/100 ml, the recovery percentage can be estimated with an accuracy of 5 per cent.

It will be seen from Table IV that with the technique used it was possible to recover approximately 100 per cent of the iodide added.

Examination was then made as to whether the analysis results were lower if no hydrogen peroxide was added to the phosphorous acids. Three sera were examined.

In contrast to the findings of Moran (1952), hydrogen peroxide did not seem to affect the effectivity of the distillation. On the other hand, it seemed to be of great

Table VII. *Significance of period distillation.*

Duration	Serum 1. PBI in µg/100 ml		Serum 2. PBI in µg/100 ml		Seru PBI µg/10
5 minutes	8.25	8.75	3.5	3.75	5.25
10 minutes	10.25	10.75	5.5	6.0	7.0
15 minutes	11.5	11.75	9.25	8.75	8.25

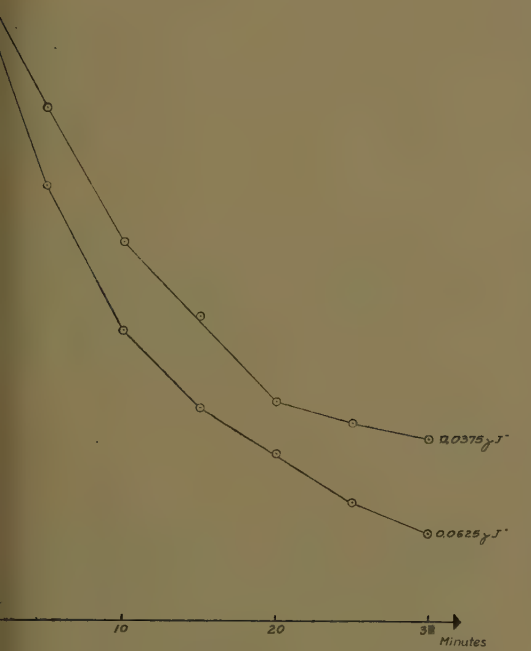
importance that there was As_2O_3 in trap solution (Table VI).

Finally, the effect of the distillation pe on the analysis results was examined three sera (Table VII).

It will be seen that a shortening o extension of the distillation period affe the analysis results. It is obvious th shortened distillation period reduces results. The reason for the fact tha extended period increases the result apparently that the distillation tempera increases gradually, so that SO_2 esc from the digested product. SO_2 is b in the trap solution, thus increasing reduction of the ceric ammonium sulp (Moran, 1952).

4. *Dependence of ceric ammonium sulp on time and temperature*

The dependence of the ceric ammo sulphate reduction on the time for two fernt amounts of iodide will be seen Fig. 3. Several writers prefer to read reaction at different times from its com cement (Connor *et al.*, 1949; Taurog *e* 1946). In the present study, we have the same method as Barker (1948) Moran (1952) and have read the reacti



.3. Dependence of reduction of ceric ammonium hate on time for two different amounts of iodide.
Abscissa: Time in minutes.
Ordinate: Extinction.
Temperature: 29° C.

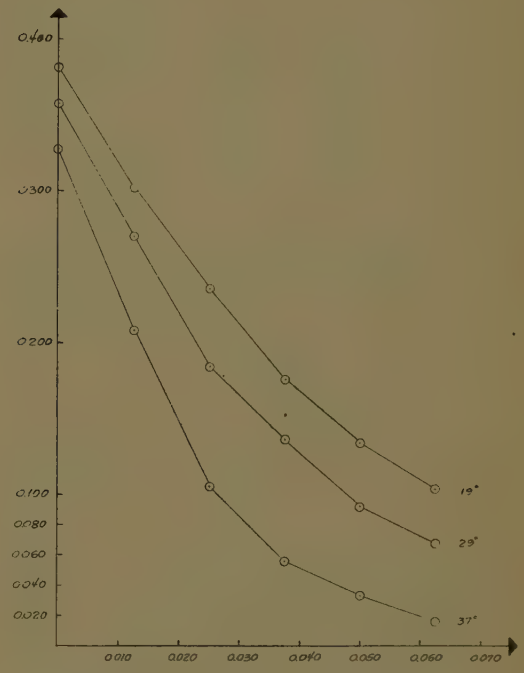


Fig. 4. Dependence of reduction of ceric ammonium sulphate on amount of iodide at three different temperatures.
Abscissa: Total amount of iodide in μg.
Ordinate: Extinction.
Reaction period: 28 minutes.

point of time, namely 28 minutes from commencement.

The reaction is sensitive to temperature. increase in temperature of 1° C increases rate of reaction by 3 per cent (Connor *al.*, 1949). In this study the temperature the water bath used did not vary more n 0.1° C. Fig. 4 shows the dependence the reaction on the amount of iodide at ee different temperatures.

The process is also sensitive to light, and s it is necessary to ensure that sunlight s not effect the reaction (Moran, 1952).

Finally, osmium, bromide and small amounts of chloride promote the speed of the reaction, whereas fluoride, larger amounts of chloride, mercury (diuretica) and silicium reduce the speed (Barker, 1948; Chaney, 1940; Russell, 1953; Salter & Mc Kay, 1944; Salter & Rosenblum, 1951).

5. *Determination of the accuracy of the method*

In order to determine the accuracy of the method in our hands, we have carried out the following tests:

Table VIII. *Results of repeated determinations on six different sera, in μg/100*

1.	5.75	5.75	6.00	5.75	5.00	5.25	5.25	6.00	5.50	m = 5.58 s =
2.	10.75	10.50	11.00	9.50	10.00	9.50				m = 10.21 s =
3.	6.75	6.25	6.25	6.50	6.25	6.25	6.00	6.25	6.50	m = 6.36 s =
	6.50	6.50								
4.	6.75	6.25	6.50	6.00	6.75	6.50	6.50	5.50		m = 6.34 s =
5.	6.00	5.50	6.50	6.75						m = 6.19 s =
6.	5.25	4.50	5.00	4.25	4.50	5.00	5.75			m = 4.89 s =

m = mean value. s = standard deviation.

(1) repeated determinations from the same serum, (2) duplicate determinations and (3) analysis of known iodine, iodide and thyroxin solutions.

(1) Repeated determinations have been made on six different sera. The results given in Table VIII. The standard deviation ($\bar{S}$) for all determinations was calculated ± 0.44 μg/100 ml according to the formula

$$\bar{S} = \pm \sqrt{\frac{s_1^2 (n_1 - 1) + s_2^2 (n_2 - 1) + \cdots + s_6^2 (n_6 - 1)}{\Sigma (n - 1)}}$$

where $s_1, s_2, \cdots s_6$ = standard deviation and $n_1, n_2, \cdots n_6$ = number of determinations on each serum.

(2) 200 routine duplicates on 200 different sera are given in Table IX. The observations are grouped according to the difference (d) between the single determinations in each duplicate. The standard deviation calculated from this material with the formula

$$\bar{S} = \pm \sqrt{\frac{\frac{d_1^2}{2} \cdot n_1 + \frac{d_2^2}{2} n_2 + \cdots + \frac{d_6^2}{2} \cdot n_6}{N}}$$

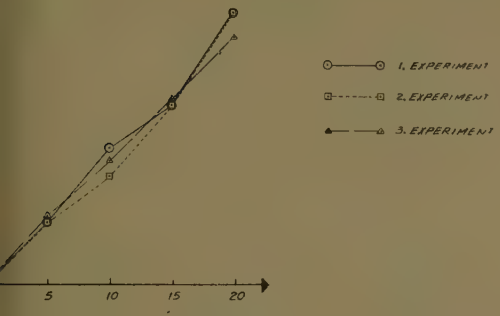
is ± 0.34 μg/100 ml. (The symbols d. n, N are explained in Table X). As these duplicates were performed after the above mentioned repetitions, the difference in $\bar{S}$ may be due to improvement in technical practice.

(3) Analyses have also been made of known iodine, iodide and thyroxin solutions, which, however, were not precipitated and washed. Very low values were found on analyses of iodide and iodine solutions, presumably because the iodine escapes during the digesting process since it is not protein-bound. The results of the analyses of three

Table IX. *Deviations in 200 dupli determinations.*

Group No.	d	No. of ca
1	0	41
2	1/4 μg/100 ml	84
3	1/2 μg/100 ml	53
4	3/4 μg/100 ml	11
5	1 μg/100 ml	4
6	1 1/4—2 μg/100 ml	7
7	More than 2 μg/100 ml	0
		N = 200

d = Difference between single determination duplicate.



g. 5. Analysis results for iodine determination of known thyroxin solutions.
Abscissa: Calculated amounts of thyroxin in μg/100 ml.
Ordinate: Amounts of thyroxin iodine found in μg/100 ml.

oups of thyroxin solutions (each consisting four solutions containing 5, 10, 15 and μg/100 ml thyroxin iodine) are shown Fig. 5. (For production of thyroxin solu-ns see page 345). It will be seen that the alyses of the thyroxin solutions do not viate from the calculated values by more an 10 per cent (except in the case of one gle determination).

SUMMARY

Mention is made of some investigations o Barker's distillation method for the termination of protein-bound iodine in um.

It was found that by adding varying ounts of KI to serum, the PBI was in-ased by amounts of 10 μg I^- per 2 ml um, whilst smaller amounts were washed ay completely. However the majority of μg thyroxin iodine could be recovered. The importance is stressed of digesting y up to the temperature where sulphuric d fumes appear, and of adhering strictly the distillation period.

An account is given of the accuracy of the method used in our laboratory. The standard deviation for repeated determination of the same serum in six different cases was $\pm$ 0.44 μg/100 ml.

ACKNOWLEDGMENT

The writer wishes to express grateful thanks to Prof. A. Querido, Academisch Zieckenhuis, Leiden, Holland, for affording facilities for studying the analysis technique.

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PAPER ELECTROPHORESIS OF PORPHYRIN PIGMENTS*

As shown by Papastamatis & Kench (1952) ıophoretic separation of porphyrins can be tained in agar gel with phosphate buffer /15, pH 8.0 and a current of 4 V/cm. By s method 1 μ of the pigments can be easured, and the recovery by extraction s 60—80 per cent.

The present report describes the use of per electrophoresis in the study of por-yrins, for which this technique has not rlier been employed.

METHOD

Electrophoretic separation of porphyrins was car-d out on Swedish Munktell 20/150 filter paper into strips 34 cm × 4 cm; five strips were used each run. Barbital-NaCl of ionic strength 0.05 8.6 and temperature 20° C ± 1° was employed buffer. The time of electrophoresis was one hour the current 14 V/cm. The rate of electro-osis was tested with glucose developed with line hydrogen phthalate. Uroporphyrin I, copro-phyrin I, coproporphyrin III and protoporphyrin solved in the buffer were each applied on the dle of a buffer-treated strip, in a suitable amount ermined by testing. Care was taken to protect materials from direct sunlight during electro-resis, a dark room being preferable. No re-geration of the apparatus was necessary. After pleted electrophoresis, the porphyrins were ated under U. V. light, cut out from the paper le wet into pieces 1 cm wide, and eluated for an hour in the dark by shaking vigorously h 10 ml of 1.5 N hydrochloric acid. After suc-of the solution through a sinter 10 G/4, the fluorescence was determined in a Farrand fluorometer, using a monochromatic filter with a maximum transmission at 365 mμ and a secondary filter transmitting above 600 mμ.

RESULTS AND DISCUSSION

Under the conditions mentioned above, good separation of uro-copro- and protoporphyrins was obtained. The method does not permit separation of the isomeric compounds I and III. The mobilities of uroporphyrin and both coproporphyrins were 12.5×10^{-5} $cm^2 \cdot sec^{-1} \cdot volt^{-1}$ and 2.38×10^{-5} $cm^2 \cdot sec^{-1} \cdot volt^{-1}$ respectively, whereas the protoporphyrin was adsorbed to the paper. Porphyrins from the urine of a patient with acute porphyria, when adsorbed to Al_2O_3 from the acidified urine and eluated with barbital buffer gave mobilities corresponding to those of the pure compounds. For clinical identification of porphyrins by paper electrophoresis, conc. NH_3 is recommended as eluant instead of barbital buffer because of its better eluating power. The recovery was 90 ± 6 per cent, 85 ± 5 per cent and 80 ± 10 per cent for uro-, copro- and protoporphyrin, respectively. In testing the effect of the voltage on the recovery, no difference could be noticed when 6.2 V/cm was compared with 14 V/cm.

In the choice of buffer, barbital appeared to be the best of several tested. Glycocoll-NaOH gave reproducible mobilities, its buffer capacity beeing small. Phosphate buffer, although it had a good buffer capacity, was

*A preliminary report was read at the meeting the Finnish Association for Clinical Chemistry Physiology on March 29, 1954.

rejected because of great differences in the mobilities obtained. In acid buffers the porphyrins tended to be adsorbed to the paper and no mobility curve could be obtained.

SUMMARY

A rapid and simple method is described for paper electrophoretic separation of porphyrin pigments. The recovery of pure uro- copro- and protoporphyrins determined fluorometrically was 90 ± 6 per cent, 85 ± 5 per cent and 80 ± 10 per cent, respectively.

Department of Medical Chemistry,
University of Helsinki, Finland.

September 10, 1955.

ACKNOWLEDGMENT

The author wishes to thank Professor C. Rim ton F. R. S., University College Hospital Me School, London, for his generosity in suppl pure specimens of the methyl esters of uro phyrin I and coproporphyrins I and III.

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Papastamatis, S. C. & Kench, J. E.: Ionopho separation of porphyrin pigments. Nature 33, 1952.

Toa Heike

CORRECTION

T. K. With: *Porphyrin Concentration* (Vol.VII, 2). P. 194, column 1, line 8, above:

Instead of 0.6535, read 0.649. The formula (4) for uroporphyrin shall be: $0.842 \cdot d_{1\,cm}$. But if — as proposed by Rimington & Sveinsson (1950) — the value 6517 is employed for both uroporphyrin I and III the formula (4) becomes: $0.832 \cdot d_{1\,cm}$.

Book Reviews

ɔGH J. Mc DONALD: *Ionography. Elecphoresis in stabilized media.*
The Year Book Publishers, Inc., Chicago, 1955.
Price: $ 6.50.

This book deals with electromigration in bilized media. The major emphasis has en placed on paper stabilized electrolytes, ıough work on ion, or particle migra-ı, in electrolytes stabilized with materials h as agar, gelatin, starch, glass beads and ny others has been reviewed. As regards perimental technique the authors state that main emphasis is placed on the horizontal er strip in a gas atmosphere, even if they ve employed almost every technique for ıducting ionographic separations. This ok gives an excellent summary of our owledge up to the present regarding elec--migration in stabilized media. The mate-l is very well arranged and clearly pre-ıted, and the coverage of the litterature ms to be very good.

S. L. S.

AN C. BURTON and OTTO G. EDHOLM: *Man in a Cold Environment. Physiological and Pathological Effects of Exposure to Low Temperatures.*
Edward Arnold (Publishers) Ltd., London, 1955.
Price: 30 sh.

This book presents in a concise and uni-l way the essential knowledge and prob-ıs pertaining to the physiology of man in old environment.

The physics entering into the various ects of heat balance, such as heat trans-ssion through clothing, tissues, etc., is en in terms simple enough so that the egrated picture is emphasized and never t in a maze of details. Also in their treat-nt of the physiological responses and adaptations to cold the authors have placed the main emphasis on those correlations which have direct thermal meaning. There are excellent treatments of the physiological problems pertaining to negative heat balance, general and local hypothermia, frost bite and freezing. The book ends with a stimulating chapter on problems for future research, and is a »must« for students of the physiology of climatic adaptation.

Many thought-provoking problems are discussed throughout the book, and the more broad-minded reader will appreciate that the authors draw extensively on the knowledge of climatic adaptation in animals to elucidate the physiology of that awesome, naked primate who has been able to penetrate the northlands only by virtue of retaining within his clothing the tropical climate of his ancestors.

P. F. S.

Clinical Pathology in General Practice.
British Medical Association, London, 1955.
Price: 21 sh.

This book is composed of a collection of articles that appeared originally in the British Medical Journal in the Refresher Course series for General Practitioners, and have been specially revised by the authors for this volume. The aim of the book is to inform the general practitioner about the kind of help he may get from the clinical laboratories and to give him some guidance in the interpretation of the reports he receives. Even if some articles are chiefly of value to british medical practitioners, *e. g.* the information given of the available laboratory facilities, the book as a whole is most valuable to practitioners everywhere and can be highly recommended.

S. L. S.

An International Congress of Clinical Chemistry is to be held in New York City, September 9—14, 1956, by authorization of the International Federation of Clinical Chemistry and the Commission of Clinical Chemistry of the International Union of Pure and Applied Chemistry. Additional information will be provided in subsequent communications or may be obtained by writing to the Congress Secretary, Dr. John G. Reinhold, 711 Maloney Bldg., Hospital of the University of Pennsylvania, Philadelphia 4, Pa., U. S. A.

Those who wish to attend are advised to make travel reservations as early as possible.

The Second International European Congress on Clinical Chemistry will take place in Stockholm, Sweden, August 19th—23rd 1957. Free papers will be read, but some actual topics in the field will be reviewed by invitated specialists. The Congress will be combined with an international exhibition of laboratory instruments. Organizing committee: Bertil Josephson M. D., chairman, address: S:t Erik's Hospital, Stockholm; Kjell Agner M. D., general secretary, Serafimer Hospital, Stockholm; Bo Norberg M. D., treasurer, Sabbatsberg's Hospital, Stockholm; Ingemar Jungner M. D., exhibition manager, Epidemiologic Hospital, Stockholm. The address of the Congress is Box 12024, Stocholm 12.

Further information about the Congress will be published and circularized later.

GUY'S HOSPITAL MEDICAL SCHOOL

Diploma in Physical Medicine Part I

The next course for this Diploma will commence during the first week of January, 1956.

Full particulars from: Physics Department, Guy's Hospital Medical School, London, S. E. 1.

INDEX OF AUTHORS

(L) Letters to the Editors, (T) Technique and Practical Problems

rengot, V. & Schmidt, H.: An examination of the relation between erythrocyte-auto-antibodies and syphilitic reagins 22

rectsen, S. R. & Thaysen, J. Hess: The excretion of urea by the human parotid gland 231

evold, R.: see Foss, O. P. 126.

rup, T., Flyger, H. H. K. & Gormsen, J.: The anticoagulant activity and toxicity of dextran sulphate and a xylan polysulphuric acid 204

rup, T. & Sterndorff, I.: The plasminogen activator in urine and the urinary trypsin inhibitor 239

gustinsson, K.-B.: A titrimetric method for the determination of plasma and red blood cell cholinesterase activity using thiocholine esters as substrates 284

ssir, O. & Hall, J.: Photo-activation as a source of error in the cephalin cholesterol flocculation test 274 (T)

dal, P. & Olhagen, B.: Protein pattern in polyp fluids and cellular infiltration of nasal polyps 35

rnesjö, K. B.: Analysis of protein-bound serum polysaccharides with anthrone reagent 147

rnesjö, K. B. Staining of protein-bound serum polysaccharides in electrophoresis strips 153

ttsen, I.: see Hedlund, P. 99 (L)

lson, L. A. & Lökk, T.: Protein studies of transsudates of the middle ear 43

ristensen, L. Korsgaard: The proteolytic activity of gastric juice 225

ristensen, L. Korsgaard: A method for determination of fibrin in plasma 246

ristensen, S., Jensen, S. Eskjær & Lundbæk, K.: The incorporation of P^{32} in phospholipid fractions in man (a preliminary report)

rkinsky, G., Diding, N. & Ouchterlony, Ö.: A new method for determination of bacterial sensitivity to chemotherapeutic agents by means of paper strips 259

n, H.: see Lund, E. 69

nielson, E.: see Sachs, B. A. 277 (T)

ling, N.: see Czerkinsky, G. 259

ljarn, L., Nygaard, O. & Sveinsson. S. L.: The determination of serum calcium 92 (T)

ksen, L.: Lead intoxication I. The effect of lead on the in vitro biosynthesis of heme and free erythrocyte porphyrins 80

Falholt, W.: Spectrophotometry on whole blood 49

Flyger, H. H. K.: see Astrup, T. 204

Foss, O. P., Askevold, R., Torp, H. E. & Sveinsson, S. L.: Conductivimetric determination of total cations in serum 126

Friis, T.: Protein bound iodine (PBI) — investigation into Barkers distillation method 336

Geill, T.: see Lund, E. 69

Georg, J.: The nitrogen clearance of the lungs 308

Gjørup, S., Poulsen, H. & Prætorius, E.: The uric acid concentration in serum determined by enzymatic spectrophotometry 201

Gormsen, J.: see Astrup, T. 204

Grotte, G.: Passage of dextran molecules of varying size from blood to lymph 59

Gräsbeck, R.: Determinations of glandular mucoprotein in the gastric juice of patients with pernicious tapeworm anemia 195 (L)

Hall, J.: see Bassir, O. 274 (T)

Hamre, L. & Kjørstad, H.: Maximum breathing capacity and vital capacity determined by means of the Krogh spirometer 300

Haugen, H. N.: The significance of azotemia in gastro-intestinal hemorrhages 75

Hedenskog, I.: see Skanse, B. 291

Hedlund, P. & Brattsen, I.: Electrophoretic analysis of human acute phase protein 99 (L)

Heikel, T.: Paper electrophoresis of porphyrin pigments 347 (L)

Hellem, A. J. & Skaug, O. E.: Paroxysmal nocturn hemoglobinuria II. Clinical features and investigations on the hemolytic tendency 121

Herrlin, K.-M. & Thilén, A.: Antihemophilic activity in Cohn's fraction I prepared from post partum serum 218

Hjort, P., Rapaport, S. I. & Owren, P. A.: Platelet accelerator: Identical to proaccelerin and adsorbed from plasma 97 (L)

Hæger, K.: Blood volume studies on candidates for thoracic surgery 175

Jacobsson, L. & Poulsen, S.: Spectrophotometric determination of creatinine in alcoholic solution 32

Jensen, S. Eskjær: see Christensen, S. 212

Karvonen, M. J. & Malm, M.: Colorimetric determination of fructose with indol 305

Keohane, K. W. & Metcalf, W. K.: On the disappearance of erythrocytes 188 (T)
Killmann, S. A. & Thaysen, J. Hess: The permeability of the human parotid gland to a series of sulfonamide compounds, paraamino-hippurate and inulin 86
Kjørstad, H.: see Hamre, L. 300

Larsen, G.: Red blood cell diameters in wet and dry preparations 62
Laurell, C.-B.: Remarks on the centrifugal force necessary for separation of plasma for fibrinogen determination 95 (L)
Laurell, C.-B.: The serum lipoproteins in slight malnutrition 257
Laurell, S.: The effect of free fatty acids on the migration rates of lipoproteins in paper electrophoresis 28
Lausen, H. H.: Determination of sodium in dried umbicial cord tissue by flame photometry 320
Linko, E. & Waris, E.: Plasma protein changes after acute myocardial infarction. Alpha2-globulin and fibrinogen 135.
Linko, E. & Waris, E.: The serum mucoprotein content and acute myocardial infarction 141
Lund, E., Geill, T., Søndergaard, E. & Dam, H.: Comparison of the prothrombin-proconvertin determinations (Owren-Aas) with "prothrombin index" obtained with the Larsen-Plum prothrombin-time method 69
Lundbæk, K.: see Christensen, S. 212.
Lura, H. Eggers: Electrochemical measurement of dissolved oxygen in biological fluids. Description of a simple microapparatus 181
Lökk, T.: see Carlson, L. A. 43

Malm, M.: see Karvonen, M. J. 305
Metcalf, W. K.: see Keohane, K. W. 188 (T)
Morrison, A. B.: see Spires, R. A. 190 (T)

Nicolaysen, R. & Ragård, R.: The efficiency of digestion in gastrectomized patients 271
Nicolaysen, R. & Ragård, R.: The calcium and phosphorus metabolism in gastrectomized patients 298
Nygaard, O.: see Eldjarn, L. 92 (T)
Nylander, G.: The electrophoretic pattern of prostatic lipid secretion in normal and pathological conditions 250
Nylander, G.: A method of estimating the activity and electrophoretic properties of "acid" prostate phosphatase 254
O'hagen, B.: see Berdal, P. 35
Ouchterlony, Ö.: see Czerkinsky, G. 259
Owren, P. A.: see Hjort, P. 97 (L)

Palmer, H. & Veflingstad, H.: Paroxysmal nocturn hemoglobinuria I. Clinical features and investigations on the hemolytic tendency 115
Pekkarinen, A. & Pitkänen, M.-E.: Noradrenaline and adrenaline in the urine. Part I 1 & Part II 8
Pers, M.: The influence of time on reabsorb from urine in colon 103
Persson, I.: Normal values for plasma fibri the age group 25—50 years 279
Pitkänen, M.-E.: see Pekkarinen, A. 1 & 8
Poulsen, H.: see Gjørup, S. 201
Poulsen, L.: see Jacobsson, L. 32
Prætorius, E.: see Gjørup, S. 201

Ragård, R.: see Nicolaysen, R. 271 & 298
Rapaport, S. I.: see Hjort, P. 97 (L)
Rosling, E. & Schoenheyder, E.: Micromethod Owrens prothrombin determination 171
Rønnow-Jessen, V.: Pentapyrrolidinium and kid function 160

Sachs, B. A. & Danielson, E.: Phospholipid de tion in paper electrophoresis 277 (T)
Schmidt, H.: see Ahrengot, V. 22
Schoenheyder, E.: see Rosling, E. 171
Skanse, B. & Hedenskog, I.: The determina of serum protein-bound iodine by alkali cineration 291
Skaug, O. E.: see Hellem, A. J. 121
Spires, R. A. & Morrison, A. B.: A simple m bolism cage for rats 190 (T)
Sterndorff, I.: see Astrup, T. 239
Storm, O.: Fibrinolytic activity in human tear
Storstein, O. & Tveten, H.: The hemodyna effect of restoring normal sinus rhythm patients with auricular fibrillation 167
Stovner, J.: Variation in the cholinesterase hibiting effect of succinylcholine 197
von Studnitz, W.: Studies on serum protein pregnancy 324
von Studnitz, W.: Studies on serum lipids lipoproteins in pregnancy 329
Støa, K. F.: Determination of thiocyanate in se with the pyridinebenzidine reaction 264
Sveinsson, S. L.: see Eldjarn, L. 92 (T)
Sveinsson, S. L.: see Foss, O. P. 126
Søndergaard, E.: see Lund, E. 69

Thaysen, J. Hess: see Killmann, S. Å. 86
Thaysen, J. Hess: see Albrectsen, S. R. 231
Thilén, A.: see Herrlin, K.-M. 218
Torp, H. E.: see Foss, O. P. 126
Tveten, H.: see Storstein, O. 167

Veflingstad, H.: see Palmer, H. 115

Waris, E.: see Linko, E. 135 & 141
Willert, B.: A new testtube method for grouping with the use of incomplete Rh bodies (saline/trypsin method). A prelimi report 111
With, T. K.: Porphyrin concentration from u violet extinction. A note on the calcul 193 (T)
Waagstein, P. H. D.: Hyperlipemia and hy glycemia in diabetes mellitus 15

INDEX OF SUBJECTS

(L) Letters to the Editors, (T) Technique and Practical Problems

e phase protein, human electrophoretic analysis of (Hedlund, P. & Brattsen, I.) 99 (L)
naline, noradrenaline and, in the urine (Pekkarinen, A. & Pitkänen, M.-E.) 111 & 112
nia, pernicious tapeworm, glandular mucoprotein in the gastric juice of patients with (Gräsbeck, R.) 195 (L)
rone reagent, analysis of protein-bound serum polysaccharides with (Björnesjö, K. B.) 147
coagulant activity and toxicity of dextran sulphate and a xylan polysulphuric acid (Astrup, T., Flyger, H. H. K. & Gormsen, J.) 204
hemophilic activity in Cohn's fraction I prepared from postpartum serum (Herrlin, K.-M. & Thilén, A.) 218
cular fibrillation, hemodynamic effect of restoring normal sinus rhythm on (Storstein, O. & Tveten, H.) 167
emia in gastro-intestinal hemorrhages, significance of (Haugen, H. N.) 75

erial sensitivity to chemoterapeutic agents by means of paper strips (Czerkinsky, G., Diding, N. & Ouchterlony, Ö.) 259
ers distillation method, investigation into, Protein-bound iodine (PBI). (Friis, T.) 336
d volume studies on candidates for thoracic surgery (Hæger, K.) 175
thing capacity, maximum, and vital capacity determined by means of the Krogh spirometer (Hamre, L. & Kjørstad, H.) 300

ium, serum, determination of (Eldjarn, L., Nygaard, O. & Sveinsson, S. L.) 92 (T)
um and phosphorus metabolism in gastrectomized patients (Nicolaysen, R. & Ragård, R.) 298
ns in serum, total, conductivimetric determination of (Foss, O. P., Askevold, R., Torp, H. E. & Sveinsson, S. L.) 126
rifugal force necessary for separation of plasma for fibrinogen determination (Laurell, C.-B.) 95 (L)
alin cholesterol flocculation test, photo-activation as a source of error in (Bassir, O. & Hall, J.) 274 (T)
notherapeutic agents, determination of bacterial sensitivity by paper strips to (Czerkinsky, G., Diding, N. & Ouchterlony, Ö.) 259

Cholinesterase activity, titrimetric determination of, using thiocholine esters as substrates (Augustinsson, K.-B.) 284
Cholinesterase-inhibiting effect of succinylcholine (Stovner, J.) 197
Cohn's fraction I prepared from post partum serum, antihemophilic activity of (Herrlin, K.-M. & Thilén, A.) 218
Colon, influence of time on reabsorbtion from urine in (Pers, M.) 103
Conductivimetric determination of total cations in serum (Foss, O. P., Askevold, R., Torp, H. E. & Sveinsson, S. L.) 126
Creatinine in alcoholic solution, spectrophotometric determination of (Jacobsson, L. & Poulsen, L.) 32

Dextran molecules of varying size, passage of, from blood to lymph (Grotte, G.) 59
Dextran sulphate and a xylan polysulphuric acid, anticoagulant activity and toxicity of (Astrup, T., Flyger, H. H. K. & Gormsen, J.) 204
Diabetes mellitus,, hyperlipemia and hyperglycemia in (Waagstein, P. H. D.) 15
Digestion in gastrectomized patients, efficiency of (Nicolaysen, R. & Ragård, R.) 271

Electrophoresis, paper, effect of free fatty acids on the migration rates of lipoproteins in (Laurell, S.) 28
Electrophoresis, paper, phospholipid detection in (Sachs, B. A. & Danielson, E.) 277 (T)
Electrophoresis, paper, of porphyrin pigments (Heikel, T.) 347 (L)
Electrophoresis strips, staining of protein bound serum polysaccharides in (Björnesjö, K. B.) 153
Electrophoretic analysis of acute phase protein (Hedlund, P. & Brattsen, I.) 99 (L)
Electrophoretic pattern of prostatic lipid secretion in normal and pathological conditions (Nylander, G.) 250
Electrophoretic properties, activity and, of "acid" prostate phosphatase, a method of estimating (Nylander, G.) 254
Erythrocytes, disappearance of (Keohane, K. W. & Metcalf, W. K.) 188 (T)
Erythrocyte-auto-antibodies and syphilitic reagins, an examination of relation between (Ahrengot, V. & Schmidt, H.) 22

Fatty acids, free, effect of, on the migration rates of lipoproteins in paper electrophoresis (Laurell, S.) 28
Fibrillation, auricular, hemodynamic effect of restoring normal sinus rhythm in patients with (Storstein, O. & Tveten, H.) 167
Fibrin, plasma, normal values of, in the age group 25—50 years (Person, I.) 279
Fibrin in plasma, a method for determination of (Christensen, L. Korsgaard) 246
Fibrinogen determination, remarks on centrifugal force necessary for separation of plasma for (Laurell, C.-B.) 95 (L)
Fibrinogen, alpha2-globulin and, plasma protein changes after myocardinal infarction (Linko, E. & Waris, E.) 135
Fibrinolytic activity in human tears (Storm, O.) 55
Fructose, colorimetric determination of, with indol (Karvonen, M. J. & Malm, M.) 310

Gastrectomized patients, efficiency of digestion in (Nicolaysen, R. & Ragård, R.) 271
Gastrectomized patients, calcium and phosphorus metabolism in (Nicolaysen, R. & Ragård, R.) 298
Gastrointestinal hemorrhages, significance of azotemia in (Haugen, H. N.) 75
Gastric juice of patients with pernicious tapeworm anemia, determination of glandular mucoprotein in (Gräsbeck, R.) 195 (L)
Gastric juice, proteolytic activity of (Christensen, L. Korsgaard) 225
Globulin, alpha2-, and fibrinogen, plasmaprotein changes after acute myocardial infarction- (Linko, E. & Waris, E.) 135

Heme and free erythrocyte porphyrins, effect of lead on the in vitro biosynthesis of (Eriksen, L.) 80
Hemoglobinuria, paroxysmal nocturn, clinical features and investigations on the hemolytic tendency I (Palmer, H. & Veflingstad, H.) 115. — II (Hellem, A. J. & Skaug, O. E.) 121
Hemolytic tendency, clinical features and investigations on, paroxysmal nocturn hemoglobinuria, I (Palmer, H. & Veflingstad, H.) 115. — II (Hellem, A. J. & Skaug, O. E.) 121
Hyperglycemia, hyperlipemia and, in diabetes mellitus (Waagstein, P. H. D.) 15
Hyperlipemia and hyperglycemia in diabetes mellitus (Waagstein, P. H. D.) 15

Indol, colorimetric determination of fructose with (Karvonen, M. J. & Malm, M.) 305
Infarction, acute myocardial, plasma protein changes after, alpha2-globulin and fibrinogen(Linko, E. & Waris, E.) 135
Infarction, acute myocardial, serum mucoprotein content and (Linko, E. & Waris, E.) 141
Iodine (PBI), protein-bound, investigation into Barkers distillation method (Friis, T.) 336

Iodine, serum protein bound, determination o alkali incineration (Skanse, B. & Heden I.) 291
Intoxication I, lead, effect of lead on the in biosynthesis of heme and free erythrocyte phyrins (Eriksen, L.) 80
Inulin, paraamino-hippurate and, permeability human parotid gland to a series of sulfona compounds (Killmann, S. Å. & Thayse H.) 86

Kidney function, pentapyrrolidinium and (Røn Jessen, V.) 160

Lead intoxication I, effect of lead on the in biosynthesis of heme and free erythrocyte phyrins (Eriksen, L.) 80
Lipid secretion, prostatic, electrophoretic patte in normal and pathological conditions lander, G.) 250
Lipids, serum lipoproteins and, studies of, in nancy (von Studnitz, W.) 329
Lipoproteins in paper electrophoresis, effect o fatty acids on the migration rates of (La S.) 28
Lipoproteins and lipids, serum, studies of, in nancy (von Studnitz, W.) 329
Lipoproteins, serum, in slight malnutrition (La C.-B.) 257

Malnutrition, serum lipoproteins in (Laurell, C 257
Metabolism cage for rats, simple (Spires, R. Morrison, A. B.) 190 (T)
Mucoprotein content, serum, and acute myoca infarction (Linko, E. & Waris, E.) 141
Mucoprotein, glandular, determination of, in g juice of patients with pernicious tapeworm mia (Gräsbeck, R.) 195 (L)
Myocardial infarction, plasma protein changes alpha2-globulin and fibrinogen (Linko, Waris, E.) 135
Myocardial infarction, acute, serum mucop content and (Linko, E. & Waris, E.) 14
Nor-adrenaline and adrenaline in urine part I karinen, A. & Pitkänen, M.-E.) 1. — II (P rinen, A. & Pitkänen, M. E.) 8
Nitrogen clearance of the lungs (Georg, J.)

Owrens prothrombin determination, microm for (Rosling, E. & Schoenheyder, E.) 1
Oxygen in biological fluids, dissolved, el chemical measurement of, description of n apparatus (Lura, H. Eggers) 181

P^{32}, incorporation of, in phospholipid fractio man (Christensen, S., Jensen, S. Eskj Lundbæk, K.) 212
Paraamino-hippurate and inulin, permeabili human parotid gland to a series of sulfon compounds (Killmann, S. Å. & Thays H.) 86

rotid gland, human, excretion of urea by (Albrectsen, S. R. & Thaysen, J. Hess) 231
rotid gland, human, permeability of, to a series of sulfonamide compounds, paraamino-hippurate and inulin (Killmann, S. Å. & Thaysen, J. Hess) 86
rtapyrrolidinium and kidney function (Rønnow-Jessen, V.) 160
osphatase, "acid" prostate, method of estimating activity and electrophoretic properties of (Nylander, G.) 254
ospholipid detection in paper electrophoresis (Sachs, B. A. & Danielson, E.) 277 (T)
ospholipid fractions in man, incorporation of P^{32} in (Christensen, S., Jensen, S. Eskjær & Lundbæk, K.) 212
osphorus, calcium and, metabolism in gastrectomized patients (Nicolaysen, R. & Ragård, R.) 298
oto-activation as a source of error in cephalin cholesterol flocculation test (Bassir, O. & Hall, J.) 274 (T)
sminogen activator in urine and urinary trypsin inhibitor (Astrup, T. & Sterndorff, I.) 239
telet accelerator, identical to proaccelerin and adsorbed from plasma (Hjort, P., Rapaport, S. I. & Owren, P. A.) 97 (L)
yps, nasal, protein pattern in polyp fluids and cellular infiltration of (Berdal, P. & Olhagen, B.) 35
ysaccharides, protein-bound serum, analysis of, with anthrone reagent (Björnesjö, K. B.) 147
ysaccharides, protein-bound serum, staining of, in electrophoresis strips (Björnesjö, K. B.) 153
rphyrin concentration from ultra violet extinction, note on calculation of (With, T. K.) 193 (T)
rphyrin pigments, paper electrophoresis of (Heikel, T.) 347 (L)
rphyrins, heme and free erythrocyte, effect of lead on in vitro biosynthesis of, lead intoxication I (Eriksen, L.) 80
gnancy, studies on serum lipids and lipoproteins in (von Studnitz, W.) 329
gnancy, studies on serum proteins in (von Studnitz, W.) 324
accelerin, platelet accelerator identical to, and adsorbed from plasma (Hjort, P., Rapaport, S. I. & Owren, P. A.) 97 (L)
state phosphatase, "acid", method of estimating activity and electrophoretic properties of (Nylander, G.) 254
static lipid secretion in normal and pathological conditions, electrophoretic pattern of (Nylander, G.) 250
teolytic activity of gastric juice (Christensen, L. Korsgaard) 225
thrombin, micromethod for Owrens determination of (Rosling, E. & Schoenheyder, E.) 171
thrombin-index obtained with the Larsen-Plum prothrombin-time method, comparison of prothrombin-proconvertin determination (Owren-Aas) with (Lund, E., Geill, T., Søndergaard, E. & Dam, H.) 69
Prothrombin-proconvertin determination (Owren-Aas), comparison of, with "prothrombin-index" obtained with the Larsen-Plum prothrombin-time method (Lund, E., Geill, T., Søndergaard, E. & Dam, H.) 69
Pyridine-Benzidine reaction, determination of thiocyanate in serum with (Støa, K. F.) 264

Red blood cell diameters in wet and dry preparations (Larsen, G.) 62
Rh-grouping, with use of incomplete Rh antibodies (saline/trypsin method), new testtube method for (Willert, B.) 111

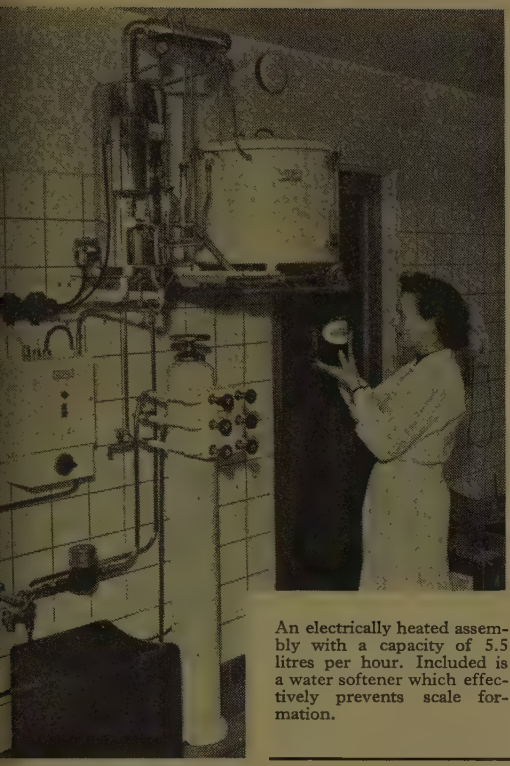
Sodium, determination of, in dried umbilical cord tissue by flame photometry (Lausen, H. H.) 320
Spectrophotometry on whole blood (Falholt, W.) 49
Spirometer, Krogh, maximum breathing capacity and vital capacity determined by means of (Hamre, L. & Kjørstad, H.) 300
Succinylcholine, variation in cholinesterase-inhibiting effect of (Stovner, J.) 197
Syphilitic reagins and erythrocyte-auto-antibodies, examination of relation between (Ahrengot, V. & Schmidt, H.) 22

Tapeworm anemia, pernicious, determination of glandular mucoprotein in gastric juice of patients with (Gräsbeck, R.) 195 (L)
Tears, human, fibrinolytic activity in (Storm, O.) 55
Thiocholine esters as substrates, titrimetric method for determination of plasma and red blood cell cholinesterase activity using (Augustinsson, K.-B.) 284
Thiocyanate, determination of, in serum with pyridine-benzidine reaction (Støa, K. F.) 264
Trypsin inhibitor, urinary, plasminogen activator in urine and (Astrup, T. & Sterndorff, I.) 239
Transsudates of the middle ear, protein studies of (Carlson, L. A. & Lökk, T.) 43

Umbilical cord tissue, determination of sodium in (Lausen, H. H.) 320
Urea, excretion of, by human parotid gland (Albrectsen, S. R. & Thaysen, J. Hess) 231
Uric acid concentration in serum determined by enzymatic spectrophotometry (Gjørup, S., Poulsen, H. & Prætorius, E.) 201
Urine, influence of time on reabsorption from, in colon (Pers, M.) 103

Vital capacity, maximum breathing capacity and, determined by means of Krogh spirometer (Hamre, L. & Kjørstad, H.) 300

Xylan polysulphuric acid, dextran sulphate and, anticoagulant activity and toxicity of (Astrup, T., Flyger, H. H. K. & Gormsen, J.) 204.



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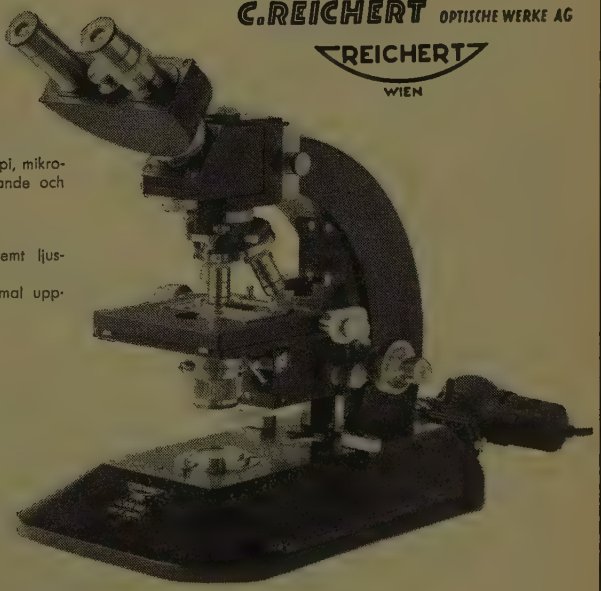

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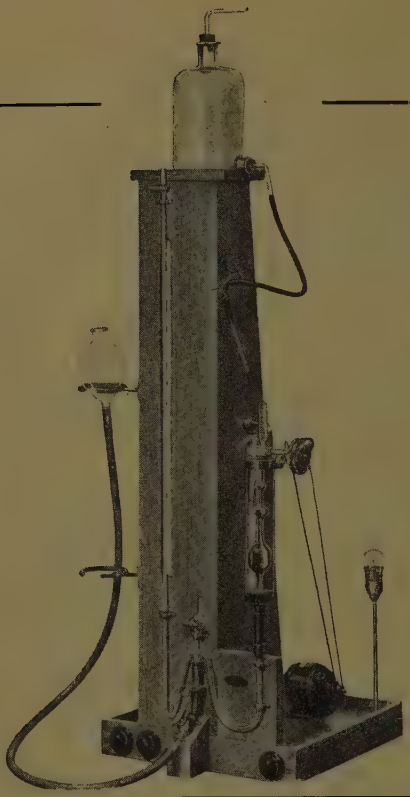

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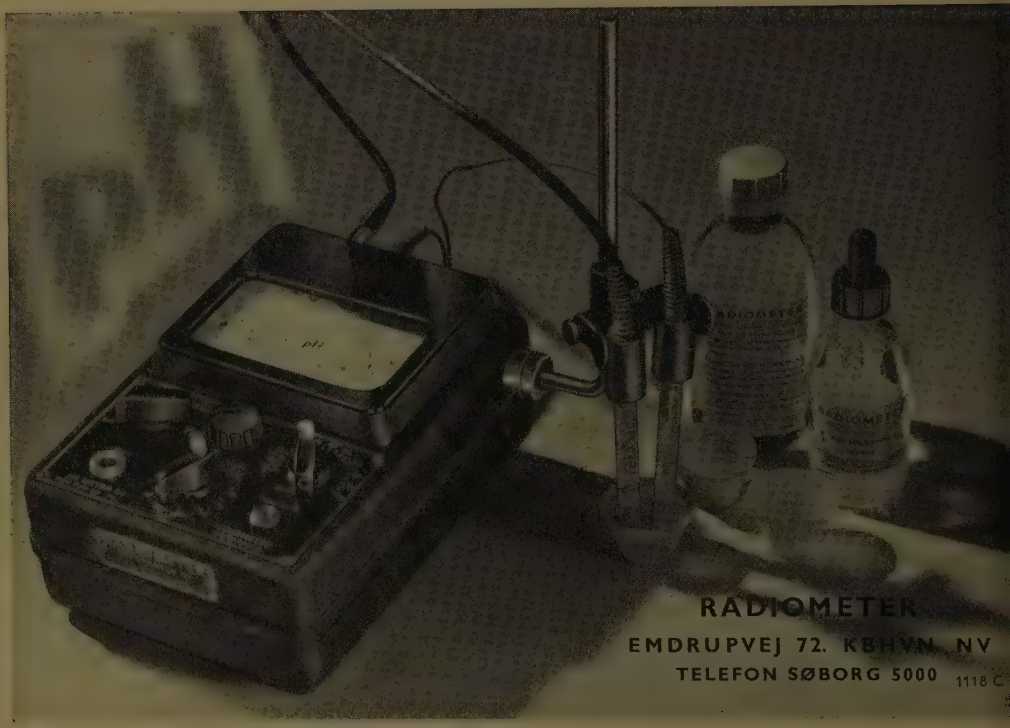

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CONTENTS OF VOLUME VII

No. 1.

A. PEKKARINEN and M.-E. PITKÄNEN: Noradrenaline and adrenaline in the urine. Part I. Their chemical determination 1
A. PEKKARINEN and M.-E. PITKÄNEN: Noradrenaline and adrenaline in the urine. Part II. Their excretion in certain normal and pathological conditions 8
P. H. D. WAAGSTEIN: Hyperlipemia and hyperglycemia in diabetes mellitus. Demonstration of arteriovenous lipoid difference 15
V. AHRENGOT and H. SCHMIDT: An examination of the relation between erythrocyte-auto-antibodies and syphilitic reagins 22
S. LAURELL: The effect of free fatty acids on the migration rates of lipoproteins in paper electrophoresis 28
L. JACOBSSON and L. POULSEN: Spectrophotometric determination of creatinine in alcoholic solution 32
P. BERDAL and B. OLHAGEN: Protein pattern in polyp fluid and cellular infiltration of nasal polyps 35
L. A. CARLSON and T. LÖKK: Protein studies of transsudates of the middle ear 43
W. FALHOLT: Spectrophotometry on whole blood 49
O. STORM: Fibrinolytic activity in human tears 55
G. GROTTE: Passage of dextran molecules of varying sizes from blood to lymph. A preliminary report 59
G. LARSEN: Red blood cell diameters in wet and dry preparations 62
E. LUND, T. GEILL, E. SØNDERGAARD and H. DAM: Comparison of the prothrombin-proconvertin determinations (Owren-Aas) with "prothrombin-index" obtained with the Larsen-Plum prothrombin time method 69
H. N. HAUGEN: The significance of azotemia in gastro-intestinal hemorrhages 75
L. ERIKSEN: Lead intoxication I. The effect of lead on the *in vitro* biosynthesis of heme and free erythrocyte porphyrins 80
S. Å. KILLMANN and J. H. THAYSEN: The permeability of the human parotid gland to a series of sulfonamide compounds, paraamino-hippurate and inulin 86

Technique and practical problems:

L. ELDJARN, O. NYGAARD and S. L. SVEINSSON: The determination of serum calcium 92

Letters to the editors:

C.-B. LAURELL: Remarks on the centrifugal force necessary for separation of plasma for fibrinogen determination 95
P. HJORT, S. I. RAPAPORT and P. A. OWREN: Platelet accelerator: Identical to proaccelerin and adsorbed from plasma? 97
P. HEDLUND and I. BRATTSTEN: Electrophoretic analysis of human acute phase protein 99
Book Reviews 101

No. 2.

M. PERS: The influence of time on reabsorption from urine in colon. Experimental studies on the conditions following ureterocolic anastomoses II 103
B. WILLERT: A new testtube method for Rh grouping with the use of incomplete Rh antibodies (saline/trypsin method). A preliminary report 111
H. PALMER and H. VEFLINGSTAD: Paroxysmal nocturnal hemoglobinuria. I. Clinical features and investigations on the hemolytic tendency 115
A. J. HELLEM and O. E. SKAUG: Paroxysmal nocturnal hemoglobinuria. II. Permeability and phosphate turnover in the red blood cells 121
O. P. FOSS, R. ASKEVOLD, H. E. TORP and S. L. SVEINSSON: Conductivimetric determination of total cations in serum 126
E. LINKO and E. WARIS: Plasma protein changes after acute myocardial infarction. Alpha$_2$-globulin and fibrinogen 135
E. LINKO and E. WARIS: The serum mucoprotein content and acute myocardial infarction 141
K. B. BJÖRNESJÖ: Analysis of protein-bound serum polysaccharides with anthrone reagent 147
K. B. BJÖRNESJÖ: Staining of protein-bound serum polysaccharides in electrophoresis strips 153
V. RØNNOV-JESSEN: Pentapyrrolidinium and kidney function 160
O. STORSTEIN and H. TVETEN: The hemodynamic effect of restoring normal sinus rhythm in patients with auricular fibrillation 167
E. ROSLING and E. SCHOENHEYDER: Micromethod for Owren's prothrombin determination 171
K. HÆGER: Blood volume studies on candidates for thoracic surgery 175
H. EGGERS LURA: Electrochemical measurement of dissolved oxygen in biological fluids. Description of a simple microapparatus 181

Technique and practical probems:

K. W. KEOHANE and W. K. METCALF: On the disappearance of erythrocytes 188
R. A. SPIRES and A. B. MORRISON: A simple metabolism cage for rats 190
T. K. WITH: Porphyrin concentration from ultraviolet extinction. A note on the calculation 193

Letters to the editors:

R. GRÄSBECK: Determinations of glandular mucoprotein in the gastric juice of patients with pernicious tapeworm anaemia 195

No. 3.

J. STOVNER: Variation in the cholinesterase-inhibiting effect of succinylcholine 197
S. GJØRUP, H. POULSEN and E. PRÆTORIUS: The uric acid concentration in serum determined by enzymatic spectrophotometry 201
T. ASTRUP, H. H. K. FLYGER and J. GORMSEN: The anticoagulant activity and toxicity of dextran sulphate and a xylan polysulphuric acid 204
S. CHRISTENSEN, S. ESKJÆR JENSEN and K. LUNDBÆK: The incorporation of P^{32} in phospholipid fractions in man. (A preliminary report) 212
K.-M. HERRLIN and A. THILÉN: Antihemophilic activity in Cohn's fraction I prepared from postpartum serum 218
L. KORSGAARD CHRISTENSEN: The proteolytic activity of gastric juice 225
S. R. ALBRECTSEN and J. HESS THAYSEN: The excretion of urea by the human parotid gland 231
T. ASTRUP and I. STERNDORFF: The plasminogen activator in urine and the urinary trypsin inhibitor 239
L. KORSGAARD CHRISTENSEN: A method for determination of fibrin in plasma.. 246
G. NYLANDER: The electrophoretic pattern of prostatic lipid secretion in normal and pathological conditions 250
G. NYLANDER: A method of estimating the activity and electrophoretic properties of 'acid' prostate phosphatase 254

C.-B. LAURELL: The serum lipoproteins in slight malnutrition 257
G. CZERKINSKY, N. DIDING and Ö. OUCHTERLONY: A new method for determination of bacterial sensitivity to chemotherapeutic agents by means of paper strips 259
K. F. STØA: Determination of thiocyanate in serum with the pyridine-benzidine reaction 264
R. NICOLAYSEN and R. RAGÅRD: The efficiency of digestion in gastrectomized

Technique and practical problems:

O. BASSIR and J. HALL: Photo-activation as a source of error in the cephalin cholesterol flocculation test 274
B. A. SACHS and E. DANIELSON: Phospolipid detection in paper electrophoresis 277

No. 4.

I. PERSSON: Normal values for plasma fibrin in the age group 20—50 years 279
K.-B. AUGUSTINSSON: A titrimetric method for the determination of plasma and red blood cell cholinesterase activity using thiocholine esters as substrates 284
B. SKANSE and I. HEDENSKOG: The determination of serum protein-bound iodine by alkali incineration. Values in normal subjects 291
R. NICOLAYSEN and R. RAGÅRD: The calcium and phosphorus metabolism in gastrectomized patients 298
L. HAMRE and H. KJØRSTAD: Maximum breathing capacity and vital capacity determined by means of the Krogh spirometer 300
M. J. KARVONEN and M. MALM: Colorimetric determination of fructose with indol 305
J. GEORG: The nitrogen clearance of the lungs 308
H. H. LAUSEN: Determination of sodium in dried umbilical cord tissue by flame photometry 320
W. von STUDNITZ: Studies on serum proteins in pregnancy 324
W. von STUDNITZ: Studies on serum lipids and lipoproteins in pregnancy 329
T. FRIIS: Protein-bound iodine (PBI) — investigation into Barkers distillation method 336

Letters to the editors:

T. HEIKEL: Paper electrophoresis of porphyrin pigments 347
Book Reviews 349

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CONTENTS

I. PERSSON: Normal values for plasma fibrin in the age group 20—50 years 279
K.-B. AUGUSTINSSON: A titrimetric method for the determination of plasma and red blood cell cholinesterase activity using thiocholine esters as substrates 284
B. SKANSE and I. HEDENSKOG: The determination of serum protein-bound iodine by alkali incineration. Values in normal subjects 291
R. NICOLAYSEN and R. RAGÅRD: The calcium and phosphorus metabolism in gastrectomized patients 298
L. HAMRE and H. KJØRSTAD: Maximum breathing capacity and vital capacity determined by means of the Krogh spirometer 300
M. J. KARVONEN and M. MALM: Colorimetric determination of fructose with indol 305
J. GEORG: The nitrogen clearance of the lungs 308
H. H. LAUSEN: Determination of sodium in dried umbilical cord tissue by flame photometry 320
W. von STUDNITZ: Studies on serum protejns in pregnancy 324
W. von STUDNITZ: Studies on serum lipids and lipoproteins in pregnancy..... 329
T. FRIIS: Protein-bound iodine (PBI) — investigation into Barkers distillation method 336

Letters to the editors:

T. HEIKEL: Paper electrophoresis of porphyrin pigments 347
Book Reviews..... 349

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